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Dissecting the molecular role of distinct binding interfaces on the retinoblastoma tumor suppressor in growth control and tumorigenesis.

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

DISSECTING THE MOLECULAR ROLE OF DISTINCT BINDING INTERFACES ON THE RETINOBLASTOMA TUMOR SUPPRESSOR IN GROWTH CONTROL AND TUMORIGENESIS

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by

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Abstract

The retinoblastoma tumor suppressor protein (pRB) functions to maintain proliferative control and act as a barrier to tumorigenesis. pRB is capable of regulating E2F transcription factors to mediate control of proliferation through transcriptional regulation of S-phase target gene expression. In addition, pRB can stabilize the CDK inhibitor p27 through an interaction with two ubiquitin ligase complexes. Further, pRB is capable of forming a unique interaction with E2F1 termed the 'specific' interaction that is capable of blocking E2F1 induced apoptosis. These functions of pRB are mediated by distinct binding interfaces and their contributions to the overall functionality of pRB are not well defined. In this thesis multiple experimental approaches are employed to study the function of the distinct binding sites in isolation to better define their functional roles. As described in chapter 2 the E2F1 'specific site' is capable of maintaining and interaction with hyperphosphorylated pRB while the E2F 'general site' is disrupted by phosphorylation. This suggests that pRB can function beyond the G_1 phase of the cell cycle to regulate E2F1 through the 'specific site'. Using a series of novel synthetic mutations of pRB we found that multiple binding sites contribute in a redundant manner to the overall cell cycle arrest ability of pRB. While, the 'general site' appears to play a critical role in the regulation of cell cycle arrest through the regulation of E2F transcription factors, the LXCXE binding cleft and the 'specific site' can function redundantly to control proliferation. A gene-targeted mouse model was developed that disrupted the 'general site' while leaving other binding sites on pRB intact. Strikingly, these mice are unable to regulate E2F target gene expression yet they maintain appropriate proliferative control in multiple cellular contexts. The maintained proliferative control by pRB appears to be largely due to the activity of p27 as disruption of E2F regulation and p27 deficiency results in loss of proliferative control and subsequent tumorigenesis. Taken together, this work defines the contribution of the distinct binding sites to the overall functionality of pRB and provides insight into the disruption of pRB in human cancer.

Keywords

pRB, E2F, E2F1, p27, retinoblastoma, cancer, apoptosis, Rb1

Co-Authorship Statement

All chapters were written by Matthew Cecchini and edited by Dr. Fred Dick.

All experiments in chapter 2 were performed by Matthew Cecchini

All experiments in chapter 3 were performed by Matthew Cecchini except for figure 3.5b which was preformed by Dr. Fred Dick

All experiments in chapter 4 were performed by Matthew Cecchini except for figure 4.6d,e which was performed by Dr. Sarah Francis. Assistance with the mouse colony and with the tumor study was provided by Dr. Daniel Passos.

In appendix 1 data presented in figure 5 and 6 was performed by Matthew Cecchini, the remainder of work was performed by members of the Rubin lab. The paper was written by Dr. Seth Rubin and Dr. Fred Dick

Dedication

Dedicated to Erin Parker

Acknowledgments

First and foremost I would like to thank Erin Parker for her support, understanding and encouragement without her this work would not have been possible. For this and many other reason too numerous to list I dedicate this thesis Erin.

I would also like to thank my family and friends for their continued support and encouragement.

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Abbreviations

pRB:	Human retinoblastoma tumor suppressor protein
pRb:	Mouse retinoblastoma tumor suppressor protein
RB1:	Human retinoblastoma tumor suppressor gene
Rb1:	Mouse retinoblastoma tumor suppressor gene
LOH:	Loss of heterozygosity
CRF:	Chromatin remodeling factor
CDK:	Cyclin Dependent Kinase
APC:	Anaphase promoting complex
CKI:	Cyclin dependent kinase inhibitor
E:	Embryonic day
ppRB:	Hyperphosphorylated pRB
RBLP:	Large pocket domain of pRB
RBC:	C-terminal domain of pRB
PBS:	Phosphate buffered saline
GSE:	Gel shift extract buffer
IPTG:	Isopropyl β-D-1-thiogalactopyranoside
ONPG:	2-Nitrophenyl-β-D-galactopyranoside
CDK2-DN:	Dominant negative CDK2
PI:	Propidium iodide
EMSA:	Electromobility shift assay
FRET:	Fluorescence resonance energy transfer
SCLC:	Small cell lung cancer
NSCLC:	Non-small cell lung cancer
ChIP-Seq:	Chromatin immunoprecipitation sequencing

Table of Contents

List of Tables

List of Figures

List of Appendices

1 Introduction

1.1 Identification of the retinoblastoma tumor suppressor as a critical element of cell cycle control

1.1.1 Cell proliferation in cancer

Proliferative control is an essential process in multicellular organisms that ensures cells only replicate at appropriate times. Evasion of these control mechanisms is one of the hallmarks of cancer and is essential for initiation and sustained growth of tumors (*1*). Many of the critical elements that regulate proliferation have been elucidated through the study of human cancers. Adult tumors are typically caused by multiple genetic alterations that disrupt many distinct cellular pathways in a heterogeneous manner across the tumor. This heterogeneity has limited the efficacy of treatment and hindered the identification of causative elements in the initiation of the cancer. In contrast, childhood tumors have been found to have 5-10 fold fewer mutations than their adult counterparts (*2*). Further, many childhood cancers arise from the inheritance of mutant proteins that result in a rapid loss of proliferative control and subsequent tumor formation. The more homogenous nature of childhood tumors has made the treatment of these cancers often much more efficacious and facilitated the identification of critical tumor suppressor pathways. The retinoblastoma tumor suppressor protein (pRB) was found to be an essential element of retinoblastoma formation. Later this was extended to show that

disruption of the pRB pathway was a central hallmark of all human cancers (*3*). However, the specific mechanisms by which pRB functions to block tumorigenesis remains in question. For this reason this thesis uses multiple approaches to investigate the tumor suppressive mechanisms of pRB.

1.1.2 Retinoblastoma

Retinoblastoma, a rare childhood cancer, occurs with an incidence between 1:15,000 and 1:20,000 live births (*4, 5*) with an average of 25 new cases identified each year in Canada (*6*). This tumor arises from the retina and typically presents in individuals before 5 years of age (*6*). Retinoblastoma typically presents in two forms: unilaterally, where only one eye is affected, and bilaterally, where both eyes are affected. In 1971, Alfred Knudsen published a pioneering study that utilized clinical data from retinoblastoma patients with either unilateral or bilateral retinoblastoma to propose a genetic basis for retinoblastoma occurrence (*7*). He suggested that the generation of retinoblastoma required two mutational events and termed this the 'two hit hypothesis' (*7*). Patients with the familial form of retinoblastoma inherited the first mutational event, making it much more likely to develop bilateral retinoblastoma after a second mutational event or 'hit'. Patients without a genetic basis required two mutational events, which made it much more likely for these patients to present with unilateral disease. This report postulated the existence of a key tumor suppressor protein whose disruption was a critical event in retinoblastoma formation.

1.1.3 Mutation of *RB1* in retinoblastoma patients

Retinoblastoma was initially associated with disruption of chromosome 13(*8, 9*) and in 1986 the retinoblastoma tumor suppressor (*RB1)* cDNA was cloned (*10, 11*). Patients with the heritable form of retinoblastoma were found to carry one mutant allele of *RB1*. The second 'hit' was shown to be the loss of heterozygosity (LOH) of the remaining wild-type *RB1* allele (*12, 13*). Shortly after the discovery of *RB1*, it was shown that proteins from DNA tumor viruses, specifically HPV-E7, SV-40 T antigen and Adenovirus E1A, could all interact with the protein product of the *RB1* gene pRB (*14-19*). These proteins are all described as oncoproteins as they have the ability to disrupt normal proliferative control mechanisms to induce cells to undergo oncogenic transformation. Further these proteins all contained a similar sequence that was required for transformation and interaction with pRB (*20, 21*). The direct interaction between the retinoblastoma tumor suppressor protein and the viral oncogenes raised the possibility that these proteins competed to regulate the same cellular pathways.

1.1.4 pRB regulates proliferation

pRB was found to be phosphorylated in a cell cycle-dependent manner at the transition between the G_1 and S phase of the cell cycle (*22*). This suggested that pRB might have a role in the regulation of the G_1-S phase transition of the cell (*22*). This is a critical decision making step for a cell, as once cells proceed into S-phase, DNA replication will begin and the cell will be committed to complete the cell cycle. pRB was found to attenuate the activity of transcription factors known as E2Fs in the G_1 phase of the cell cycle and this inhibition was relieved upon S-phase entry (*23-25*). E2Fs induce a

transcriptional program at the G_1-S transition that functions to drive the entry into S-phase and initiate DNA synthesis (*26*). The importance of E2Fs in transcriptional control of the cell cycle control is highlighted by the ability of E2F1 to drive the entry of cells into S-phase from quiescence (*27*). This described a functional network of the G_1 checkpoint with pRB negatively regulating the ability of E2Fs to activate the transcription of key S-phase target genes to control S-phase entry.

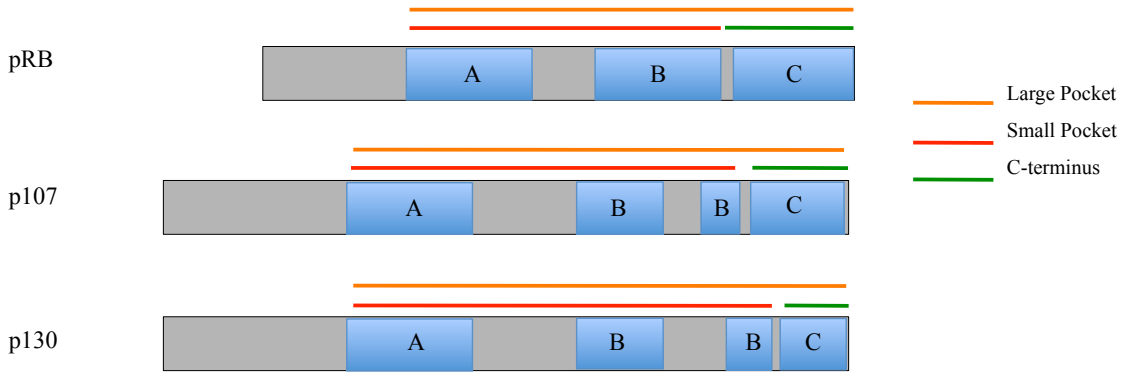
1.1.5 Summary

The study of human tumors has led to the identification of pRB as a central regulator of the G_1-S transition of the cell cycle. This regulation appears to be imparted upon E2F transcription factors and has highlighted a transcriptional mechanism of cell cycle control. While, the *RB1* gene is disrupted in retinoblastoma the majority of human cancers contain functional pRB that is inactivated through deregulation of upstream regulators of pRB activity (*28*). Small cell lung cancer is a notable exception as 90% of cancers have inactivation of the *RB1* gene (*28-30*). Interestingly, inactivation of *RB1* is a rare event in non-small cell lung cancer with only 10% of cancers having inactivation of pRB (*28, 29, 31, 32*). The apparent selection for inactivation of *RB1* in a subset of cancers suggests a complex role for pRB in tumorigenesis that extends beyond the simple negative regulation of E2Fs at the G_1-S transition. However, current models of pRB function suggest that E2F regulation is a central element of pRB function and is critical for maintaining proliferative control.

1.2 Pocket protein family

1.2.1 The pocket domain

pRB is a member of the pocket protein family that also includes p107 and p130 (*33, 34*). The pocket protein name is derived from the fact that all members share a well-conserved pocket domain (*33*). The pocket domain can be further seperated into the small and large pocket domains (Fig. 1.1). The large pocket is required for full growth suppression activity and is sufficient to complement the tumor suppressive properties of pRB when expressed in place of the full-length protein (*35, 36*). The small pocket, in contrast, is defined as the minimal domain required for interaction with the viral oncogenes (*37*). The crystal structure of the small pocket has been determined (Fig. 1.2), and the domain consists of two halves defined as A and B, which both adopt cyclin-like folds (*38, 39*). These two halves interact with one another to form a dumbbell shaped globular domain (*38, 39*). This small pocket domain is well conserved between the pocket proteins, though there are some subtle differences. Specifically, p107 and p130 both contain relatively large insertions in the B region of the small pocket (Fig. 1.1). Furthermore, the flexible linker that connects the two halves of the pocket is significantly longer in the p107 and p130 proteins. The functional implications of these alterations are not well understood, however, there are some reports that suggest that these regions allow for distinct regulation and functionality of p107 and p130 (*33*).



Domain structure of pRB, p107 and p130. The relative size and location of the A and B regions of pocket are defined along with the C-terminus of the pocket proteins. The large pocket is denoted in orange, the small pocket in red and the C-terminus is shown in green. .

Figure 1-1 Domain Structure of the pocket protein family

The pocket proteins are highly conserved as the majority of multicellular organisms contain a close homologue to the pocket protein family of proteins (*40*). While, mammals contain a complement of three pocket proteins lower organisms contain fewer pocket proteins. *Drosophila* has only two proteins termed RBF1 and RBF2 and *Caenorhabditis elegans* contains only a single homologous protein lin35. These proteins appear to share similar roles as disruption of these proteins contribute to ectopic proliferation in *Drosophila* and *C. elegans (40).* Multi-component complexes have been identified that contain pocket protein homologues that are thought to be critical to transcriptional regulation in *Drosophila* and *C. elegans.* These complexes termed dREAM/MMB in *Drosophila* (*41, 42*) and DRM in *Caenorhabditis* (*43*) contain pRB homologues, E2F/DP proteins, orthologues of the histone binding RbAp46/RbAp48, MYB and a series of MYB interacting proteins (*40*). These large protein complexes are thought to function as a critical element of transcriptional control in *Drosophila* and *Caenorhabditis.* In mammals these protein complexes are also conserved but they do not contain pRB but rather multi-subunit complexes have been identified with p107 and p130 that contain MYB proteins and other associated proteins (*44-46*). Interestingly, pRB has not been associated with these complexes in mammalian cells. This suggests that p107/p130 appear to have maintained the more evolutionary conserved roles of the pocket proteins. Further, p107 and p130 share more homology to *Drosophila* and *Caenorhabditis* pocket proteins than pRB (*40*). The pocket proteins are an evolutionary conserved group of proteins of which p107 and p130 appear to share similar functionality to the ancestral protein while pRB has diverged such that it does not appear to participate in the same evolutionary conserved complexes.

1.2.2 The pocket proteins regulate the activity of E2F transcription factors

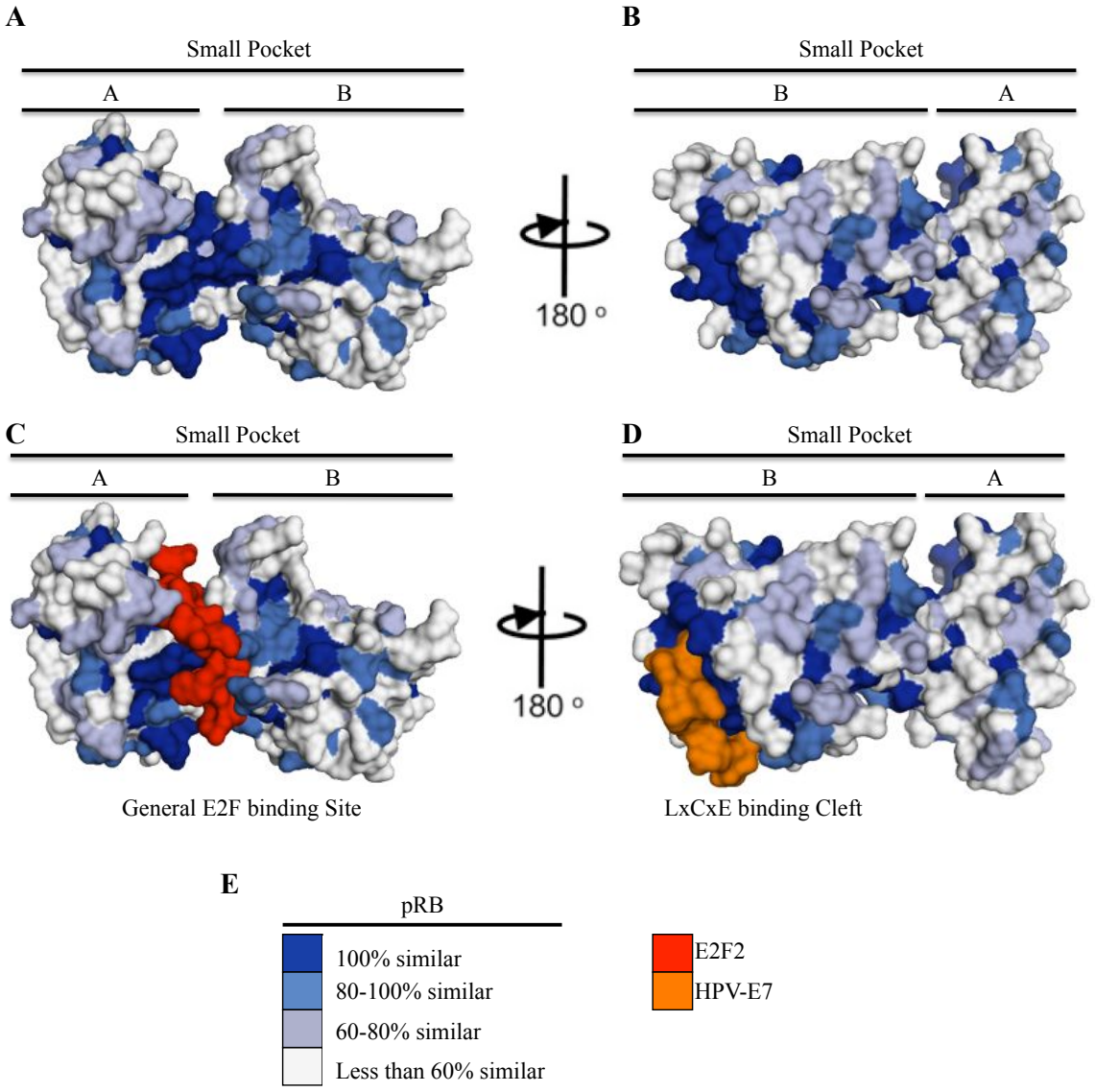
The large pocket domain has the ability to interact with E2F transcription factors and regulate their transcriptional activity. This is mediated by an interaction between the transactivation domain of E2Fs with the pocket domain of pRB (*47*). The direct interaction of pRB with the transactivation domain blocks the ability of E2Fs to activate transcription (*48*). The complex of pRB and E2F has been crystallized and defines the molecular contacts between pRB and E2Fs (*47, 49*). Short peptides derived from the transactivation domain of E2Fs were found to interact with the pocket domain at the interface between the A and B halves of the pocket domain (Fig. 1.2c). Figure 1.2 displays the small pocket domain of pRB colored by the relative conservation. The interface between the A and B halves in figure 1.2a is one of the most conserved surfaces of pRB and mediates the interaction with E2F transcription factors. The contacts between E2F and pRB occur primarily through interaction with the A region of the pocket and are mediated in part by a series of well-conserved basic residues that contact the largely acidic E2F transactivation domain shown in figure 1.3. Taken together the pocket proteins have a well conserved cleft that is formed by the A and B halves of the pocket domain that uses a series of electrostatic contacts to interact with the acidic transactivation domain of E2F transcription factors.

The small pocket is not sufficient to interact with E2Fs *in vivo*, as the C-terminal domain is also required. The C-terminal domain is thought to be largely unstructured but appears to have the capacity to form defined structures when complexed with other

interacting proteins (*50*). It has been shown that the C-terminal region contains multiple contact sites that stabilize the interaction between pRB and E2Fs (*50, 51*). Furthermore, while the C-terminal domain is not as highly conserved as the small pocket domain the essential contact regions with E2Fs appear to be conserved (*50*). The pocket domain is a conserved feature of this family of proteins and it functions in part to mediate the interaction with E2Fs through a series of distinct contact sites found throughout the large pocket region of pRB.

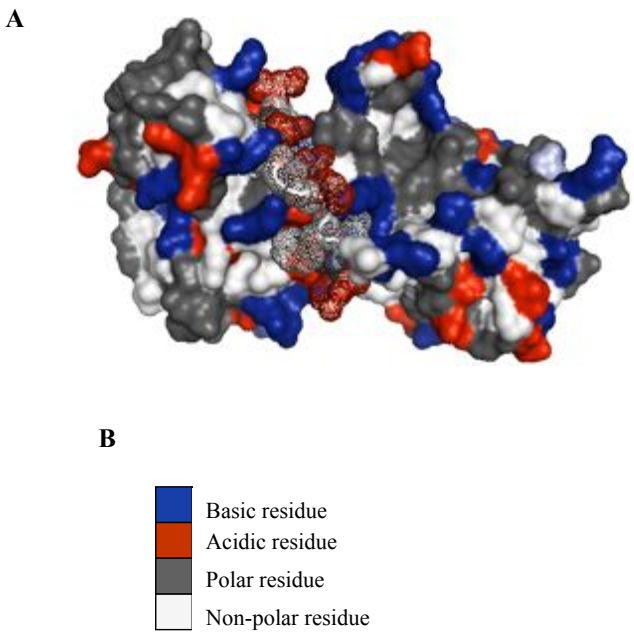
1.2.3 Interaction with chromatin remodeling factors through the LXCXE binding cleft

The other highly conserved binding interface is the LXCXE binding cleft (Fig 1.2b,d). This binding site is located on the B-half of the pocket domain on the opposite face from the E2F binding site. This region was initially found to interact with the viral oncoproteins and co-crystallization studies have confirmed that multiple viral oncogenes including E1A, HPV-E7 (Fig. 1.2d) and T antigen interact at this binding site (*39, 52*). A shallow hydrophobic binding cleft is found in this region that interacts with a host of cellular proteins in addition to the viral oncoproteins (*39*). To date there are over 25 confirmed proteins that interact with the LXCXE binding cleft of pocket proteins (*53*). The majority of these proteins are involved in modifying chromatin structure. Notable examples of these proteins are HDAC 1 (*54*) and Suv39h1 (*55*), both of which function to produce repressive chromatin environments. HDAC1 is a histone de-acetylase that functions to remove acetyl groups from lysine residues on histone tails. This promotes the



The E2F binding site and LxCxE binding cleft are conserved elements of the pocket proteins. (A-D) The RB crystal structures (A,C PDB:1N4M) (B,D PDB:1GUX) were colored based upon residue similarity across the pRB homologues from human, newt, chicken, fruit fly, maize, human p107 and human p130. As shown in (E) the darkest shade of blue indicates residues with similar properties are conserved in all pRB homologues and white indicates that less than 60% of the aligned residues are similar at that position. (C) Depicts the co-crystallized E2F2 peptide bound at the interface of the A and B regions of the small pocket. Depicted in (D) is the co-crystallized HPV-E7 derived peptide bound to the LXCXE binding cleft on the opposite face of the small pocket domain in the B region.

Figure 1-2 Conservation of the E2F and LXCXE binding sites



The cleft between the A and B regions of the small pocket domain of pRB is highly basic and interacts with a series of conserved acidic residues in the transactivation domain of E2Fs. (A) The crystal structure of pRB (PDB:1N4M) with co-crystallized E2F2 peptide is colored based upon the charges of individual residues. As shown in (B) basic residues are colored blue, acidic residues red, polar residues grey and non-polar residues white.

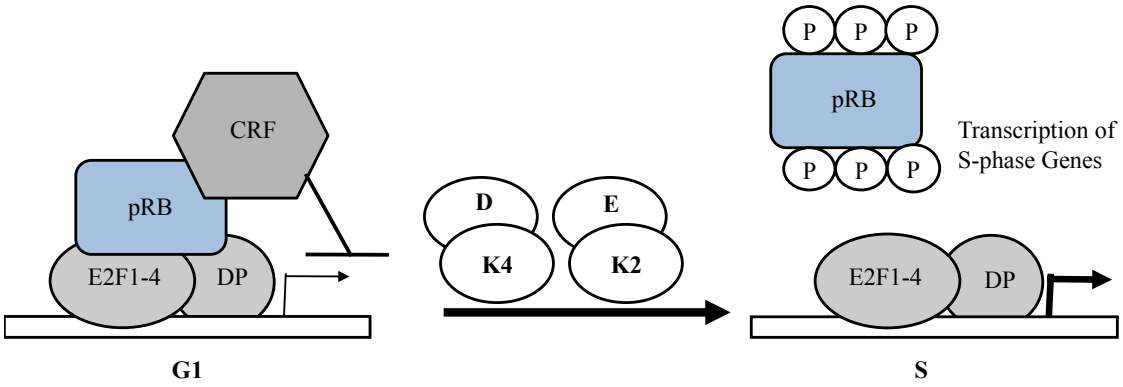
Figure 1-3 Electrostatic interface between pRB and the E2F transactivation domain

formation of a closed heterochromatin conformation that blocks gene expression (*56*). Suv39h1, in contrast, is capable of methylating lysine 9 on histone H3. This modification recruits HP1 that promotes the formation of heterochromatin that is not accessible for transcription (*55*). Pocket proteins are proposed to utilize this binding cleft to recruit chromatin-remodeling factors (CRFs) to actively repress E2F target genes through the formation of a closed chromatin environment.

In addition to the interaction with CRFs to actively suppress the expression of E2F target genes the LXCXE binding cleft has been implicated in the maintenance of genomic stability. pRB has the ability to interact with components of the condensin complex which have a critical role in maintaining correct genomic architecture and stability (*57*). Mice with targeted disruption of the LXCXE cleft in pRB were defective in forming complexes between pRB and condensin II and were observed to have chromosome stability defects akin to those observed in condensin II depleted cells (*58*). Thereby, the well-conserved LXCXE binding cleft has the ability to interact with distinct signaling elements to affect not only E2F target gene regulation but also the maintenance of genomic stability through the interaction with condensin complexes.

1.2.4 Current model of proliferative control by the pocket proteins

A model for pocket protein function has been derived that is shown in figure 1.4. This model integrates the ability of pocket proteins to interact with both E2F and chromatin remodeling factors. The pocket proteins are unable to interact with DNA, however, upon interaction with E2Fs the proteins are capable of interacting with E2F



Model of pRB function in the regulation of the G1-S phase transition. 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 including CDK4 (K4) with Cyclin D (D) and CDK2 (K2) with Cyclin E (E). These kinases extensively phosphorylate pRB and mediate the release of E2F/DP complexes. The free E2Fs activate the transcription of E2F target genes to drive the progression into S-phase and through the remainder of the cell cycle.

Figure 1-4 Transcriptional control of proliferation by pRB

target sequences (*25, 59*). In turn, the pocket proteins can act as adapter proteins to recruit distinct chromatin remodeling factors to these E2F target genes (*59*). Thereby, pRB and other pocket proteins function by not only regulating the activity of E2F transcription factors, but also by actively repressing E2F target genes through the recruitment of CRFs to produce repressive heterochromatin states that further repress expression of these genes.

1.3 Regulation of pocket protein activity

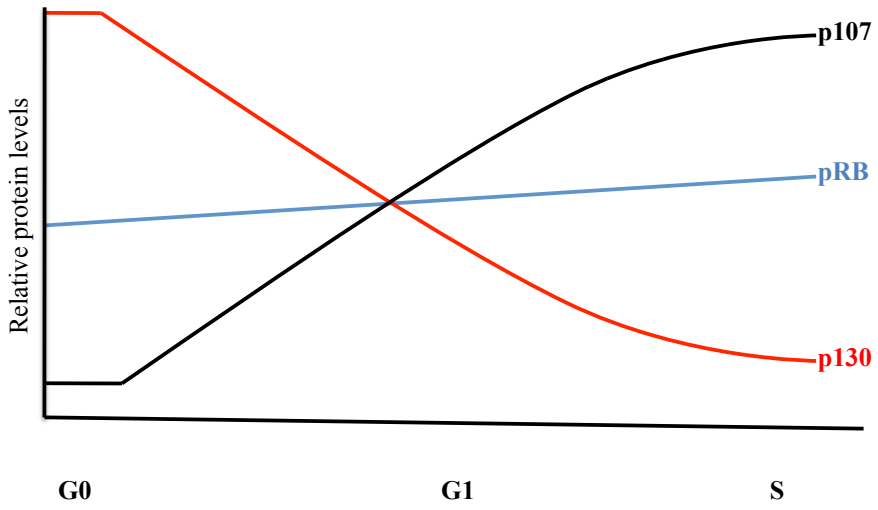
1.3.1 Differential expression mediates distinct roles of the pocket proteins

The pocket proteins share a similar mechanism of regulating the transcription of E2F target genes, however, they appear to have distinct cellular roles. This differential activity is due in large part to their differential expression (*33*). As depicted in figure 1.5 pRB is a highly stable protein that is expressed at similar levels throughout the cell cycle and its activity is primarily regulated by phosphorylation (*60*). In contrast, p107 is an E2F target gene, and as such, its expression correlates with E2F transcriptional activity (*61, 62*). The expression of p107 is low in quiescent cells but increases sharply in S-phase after activation of E2Fs. p130 is expressed primarily in quiescent and differentiated cells with correspondingly low levels in proliferative cells (*63*). The low levels of p130 are due in part to its degradation in proliferative cells (*33*). Specifically the E2F target gene Skp2 is capable of interacting with and targeting phosphorylated p130 for degradation (*64*). In cultured cells the pocket proteins p107 and p130 have an inverse relationship with the expression limited to proliferating and non-cycling cells respectively. In contrast pRB is

maintained throughout the cell cycle and is expressed in both proliferating and non-cycling cells.

1.3.2 Post-translational modifications of the pocket proteins

The pocket proteins are relatively stable with considerable half-lives that extend beyond 10h for pRB (*65*). This stability results in proteins that persist through multiple stages of the cell cycle. Thereby, the activity of pocket proteins is governed largely through post-translational modifications, specifically, phosphorylation. In the G_1-phase of the cell cycle, pocket proteins exist in a hypophosphorylated state. Mitogenic signaling results in the activation of cyclin-dependent kinases (CDKs) that phosphorylate pRB and other pocket proteins to maintain the proteins in a hyperphosphorylated state through the remainder of the cell cycle (*60, 66*). Initially, cyclin D CDK4 complexes are activated and utilize a docking site found in the C-terminus of pRB to phosphorylate it (*67*). Complete phosphorylation of pRB requires cyclin E CDK2 activity, which phosphorylates the remaining sites on pRB (*68*). While p107 and p130 are phosphorylated in a similar manner the differences in their structures allows for activities that are not observed in pRB. Specifically, the large spacer region between the A and B halves of the pocket domain along with the insertion in the B domain contain high affinity sites for cyclins that allow p107 and p130 to act as inhibitors of the cyclin-dependent kinases and potentially provide an additional mechanism of cell cycle control (*69, 70*).

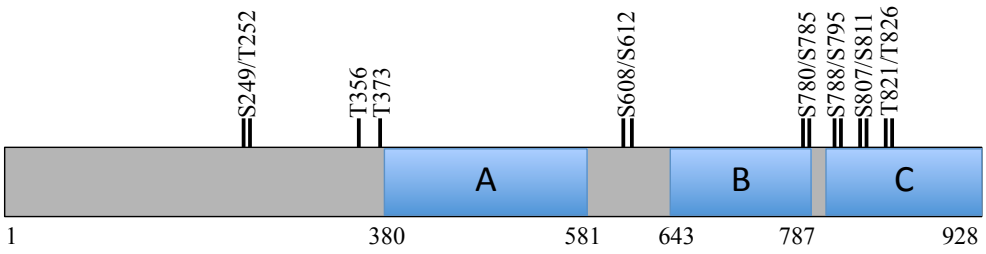


Model of relative protein levels for the three pocket proteins throughout the cell cycle adapted from Classon and Dyson, 2001. pRB shown in blue is expressed at relatively constant levels throughout the cell cycle, p107 shown in black is expressed predominately in proliferating cells in late G1 and S-phase, while p130 is expressed at highest levels in non-proliferating cells and at very low levels in proliferating cells.

Figure 1-5 Levels of the pocket proteins throughout the cell cycle

1.3.3 Disruption of E2F binding by phosphorylation

pRB contains 14 putative consensus CDK phosphorylation sites distributed throughout the protein and at least 10 distinct phosphopeptides of pRB have been identified (*71, 72*). These sites are shown in figure 1.6 and localize predominantly to regions of the pRB that are thought to lack intrinsic structure (*39, 50*). The phosphorylation sites localize primarily to the flexible linker between the A and B halves of the pocket domain, the disordered C-terminal domain, and the N-terminal domain of pRB as shown in figure 1.6 (*39, 50, 73*). Interestingly, the majority of the predicted sites are distributed in pairs with two phosphorylation sties found in close proximity to one another. Phosphorylation of pRB results in conformational changes in these flexible domains that are enriched with CDK consensus sites (*74*). These conformational changes function in part to obscure the E2F binding site that is mediated by the cleft between the A and B halves of the pocket domain (*74*). Specifically, phosphorylation of the linker between the A and B region results in a conformational change that leads to an interaction between the phosphorylated linker and the cleft formed by the A and B halves of the pocket domain (*74*). This obstructs the E2F binding site and blocks the interaction between pRB and the transactivation domain of E2Fs (*74*). pRB is extensively phosphorylated in regions that flank the well-structured pocket domain to cause a concerted change in structure that obscures the E2F binding site and frees E2Fs from the negative repression by pRB.



Relative locations of CDK phosphorylation sites on pRB. The majority of CDK sites are found in pairs outside of the structured regions of the small pocket domain in the N-terminal domain, the linker between the A and B regions and in the C-terminal region of pRB.

Figure 1-6 CDK phosphorylation sites on pRB

1.3.4 Protein phosphatases are capable of reactivating phosphorylated pRB

At the transition between metaphase and anaphase, there is a sharp decrease in the activity of CDKs that results from the degradation of cyclin B by the anaphase-promoting complex (APC) (*75*). At this time pRB must be returned to a hypophosphorylated state in the G_1 phase of the cell cycle. This is largely mediated by the active dephosphorylation of pRB by phosphatases including protein phosphatase 1 (PP1), which dephosphorylates pRB beginning in anaphase (*76, 77*). When environmental conditions are unfavorable for cell division the cell is capable of inducing an acute arrest of proliferation that also results in the reactivation of pRB to a hypophosphorylated state. 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 (*78, 79*). Upon DNA damage PP1 is activated to promote the dephosphorylation of pRB to a hypophosphorylated state that can maintain the interaction with E2Fs and block their transcriptional activity to mediate an acute arrest of the cell cycle (*80*). In addition protein phosphatase 2 also interacts with and dephosphorylates pRB in some cellular contexts (*81*). The relative contribution of PP1 and PP2 mediated effects on pRB phosphorylation are not well described and warrant further investigation. It is apparent that a robust regulatory network exists whereby pRB activity is modulated by its phosphorylation state that is controlled by the opposing activity of both kinases and phosphatases.

1.3.5 Regulating the activity of CDKs

The progression through the cell cycle is largely governed by the activity of CDKs that function to phosphorylate specific targets including pRB in a timely fashion to mediate the ordered progression through the cell cycle. Mammalian cells contain at least 11 distinct CDKs that are all activated by regulatory cyclin subunits (*82*). In addition to requiring a cyclin subunit for activity CDKs are regulated by multiple mechanisms that include, phosphorylation on activating or inhibiting sites and the interaction with cyclin dependent kinase inhibitors (CKIs) (*83*). The CKIs have a critical role in regulating cell cycle progression as they represent a means by which the activity of CDKs can be blocked abruptly in response to cellular cues such as DNA damage or developmental signals. The CKIs can be grouped into two distinct classes, the Cip/Kip proteins include $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ and the INK4 family includes $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ (*84*). The INK4 class of inhibitors function to inactive CDK4 and CDK6 complexes by blocking the association of the kinases with the regulatory cyclin D subunit. The Cip/Kip family interacts with both the cyclin and CDK subunits to regulate the activity of a broad class of cyclin CDK complexes (*83*)

The INK4 family of inhibitors requires the activity of Cip/Kip proteins to induce a cell cycle arrest (*85*). This is due to the fact that these proteins function by inhibiting CDK4/6 cyclin D complexes and releasing the sequestered Cip/Kip proteins bound to CDK4/6 to inactivate CDK2 complexes. (*85*) The Cip/Kip proteins are potent anti-proliferative factors that are capable of restraining proliferation in multiple contexts including development, differentiation and in response to cellular stresses. These distinct

contexts of cell cycle arrest typically induce only a single Cip/Kip factor. p21 is a critical target of p53 and induces a cell cycle arrest in response to cellular and genotoxic stresses (*86*). p27 in contrast is activated in response to lack of mitogens or in the induction of a quiescent state (*87*) and p57 typically mediates developmentally mediated cell cycle arrest paradigms (*88*). These proteins act to integrate distinct signals to induce a cell cycle arrest through the inhibition of CDK complexes.

1.4 Unique and overlapping functions of the pocket proteins

1.4.1 Disruption of *Rb1* in mice

Discerning the functional role of the distinct pocket proteins has been greatly aided by the use of gene-targeted mouse models to specifically disrupt individual pocket proteins. Genetic disruption of the mouse *Rb1* gene to produce $Rb1^{-/-}$ mice results in embryonic lethality between embryonic day (E) 13.5 and E15 (*89*). The $Rb1^{-/-}$ embryos have increased proliferation and apoptosis in the central nervous system (CNS) and the peripheral nervous system (PNS) (*89*). These mice also have defects in hematopoiesis and altered development and proliferation of the lens (*89-92*). The inappropriate proliferation observed in $Rb1^{-/-}$ mice can be partially rescued by combined disruption of E2F1 or E2F3, which suggests that E2F regulation is a critical function of pRB (*93-95*). The ectopic proliferation and apoptosis observed was thought to highlight a critical role for pRB in maintaining appropriate growth control in distinct developmental contexts.

However, many of the defects in *Rb1*$^{-/-}$ mice were later found to be secondary to proliferative defects that occur in the placenta of these mice (*96*).

Specifically, the trophoblast cells found within the labyrinth layer of the placenta were found to be hyperproliferative in *Rb1*$^{-/-}$ embryos (*97*). This over-proliferation results in a decrease in the space between the maternal and fetal blood supply (*96*). This in turn was found to result in decreased nutrient transport to the embryos (*96*). To investigate phenotypes of *Rb1*$^{-/-}$ mice that are independent of the placental defect *Rb1*$^{-/-}$ mice were produced with normal placenta using tetraploid aggregation (*97*). The provision of the normal placenta was found to rescue many of the phenotypes associated with loss of *Rb1 (97).* Most notably the mice were no longer embryonic lethal between E13.5-E15.5 but rather could survive until birth (*96*). The defects in hematopoiesis and apoptosis in the CNS were not observed in the rescued *Rb1*$^{-/-}$ animals (*96*). However, the excess proliferation in the CNS and the lens was observed in the rescued animals suggesting that these defects occurred independently of the placental defects (*96*). The mice, however, died shortly after birth due to defects in skeletal muscle formation (*96*). This defect results in significant disruption of the diaphragm that prevents the lungs of newborn *Rb1*$^{-/-}$ animals from inflating and resulted in an inability for the mice to respire. Experiments using conditional deletion of pRB in myoblasts suggest that the defect in skeletal muscle results from an inability of the *Rb1*$^{-/-}$ cells to terminally differentiate into multinucleated myotubes (*98*). Fibroblasts generated form *Rb1*$^{-/-}$ embryos also display significant defects in proliferative control. Specifically the fibroblasts have a shorter G_1 phase of the cell cycle, with a coincident reduction in cell size (*99*). While the cells remain sensitive to

serum starvation they are unable to respond to ectopic arrests induced by p16 (*100*) and TGFβ (*101*).

Taken together mouse models of pRB have defined an essential role for pRB in mammalian development. Specifically pRB function is required for proper proliferative control, placental development and muscle differentiation. Many cell types are able to proliferate and respond normally in distinct developmental contexts, as *Rb1*$^{-/-}$ animals are viable until birth if supplied with a normal placenta. Given the essential role for pRB in tumorigenesis and the postulated role in the regulation of the G_1-S transition the development of these mice suggests that in some contexts other pathways can function in the absence of pRB to maintain cell cycle control.

1.4.2 Redundancy in the pocket protein family

pRB appears to have a clear role in regulating a distinct set of developmental processes as observed from the *Rb1*$^{-/-}$ mice. However, proliferative control is maintained in many tissues and death in animals with a normal placenta is the result of defective differentiation of muscle cells. The maintenance of proliferative control in *Rb1*$^{-/-}$ mice appears to be due in part to the activity of the other pocket proteins p107 and p130. Loss of pRB results in a deregulation of E2F target gene expression that induces the expression of p107 which is itself an E2F target gene (*102*). The increased levels of p107 can allow for compensation for loss of pRB in many contexts. Combined disruption of pRB and p107 or pRB and p130 results in more severe apoptotic and proliferative defects that result in earlier embryonic lethality between E11 and E13 (*103, 104*). Importantly loss of p107 or p130 alone does not alter the viability of mice in a mixed genetic background

(*105*). Combined disruption of both p107 and p130 results in neonatal lethality with severe defects in bone development that results in shorter bones (*105, 106*). This suggests that the pocket proteins control partially overlapping pathways and in some circumstances function to compensate for the loss of other pocket proteins.

To further test the compensation between pocket proteins, fibroblasts were generated that disrupted pRB, p107 and p130, called TKO cells (*107, 108*). These fibroblasts were generated from the differentiation of directly targeted ES into TKO fibroblasts (*107, 108*). The TKO cells are defective for proliferative control and do not arrest in the G_1 under a variety of conditions (*107, 108*). However, more recently TKO embryos have been generated and survive until days 9-11 of gestation (*109*). Further the embryos and cultured TKO cells are capable of exiting the cell cycle in G_1 and differentiating into multiple epithelial and neural lineages (*109*). This suggests that in some contexts cell cycle exit can occur in the absence of pocket protein activity however, the mechanism by which this may occur is still unclear.

1.4.3 A unique role for pRB in cancer

Loss of pRB in the retina results in the generation of retinoblastoma early in life. Initial efforts using the mouse model of *Rb1* disruption investigated whether a similar effect would be observed in pRB null mice. In contrast to humans the $Rb1^{+/-}$ mice do not develop retinoblastoma but rather develop pituitary tumors that arise from the intermediate lobe of this gland. (*89*) $Rb1^{+/-}$ mice typically develop tumors by one year of age in either the intermediate lobe of the pituitary gland or less frequently in the thyroid gland (*110*). These tumors display loss of heterozygosity (LOH) of the remaining wild

type allele of *Rb1* to produce a tumor that is nullizygous. Interestingly disruption of other cell cycle regulators such p27 or p18 also results in pituitary tumors in the intermediate lobe (*111-114*). This suggests that this region may be uniquely susceptible to loss of proliferative control that gives rise to the observed tumors. Furthermore, the intermediate lobe is rudimentary and likely non-functional in humans which may explain the fact that pRB loss in humans does not induce pituitary tumors (*115*).

In contrast, the induction of retinoblastoma in mice requires disruption of both pRB and p107 as $Rb1^{-/-}$ $Rbl1^{-/-}$ chimeras or mice with deletion of pRB and p107 in the retina develop spontaneous retinoblastomas (*116, 117*). This further supports the compensatory role of p107 in the absence of pRB. p107 and p130 themselves are rarely disrupted in human cancers and mice lacking p107 gene *Rbl1* or the p130 gene *Rbl2* are not prone to tumors (*118*). Further, $Rbl1^{+/-}$ $Rbl2^{-/-}$ and $Rbl1^{-/-}$ $Rbl2^{+/-}$ mice are not tumor prone suggesting a unique role for the remaining pRB in tumor suppression (*34*). Taken together this suggests that pRB has a unique role in tumorigenesis but the other pocket proteins can function in the absence of pRB in certain contexts to maintain proliferative control. Given the ability of all pocket proteins to interact with E2F transcription factors the mechanistic basis for the unique role of pRB in tumorigenesis is not clearly defined.

1.5 E2F family of transcription factors

1.5.1 Division of labor in the E2F family

In a similar manner to the pocket proteins, E2Fs consist of a family of proteins that share many common features but also have unique functions that differentiate the

individual E2Fs. There is a division of labor in pocket protein E2F interactions as specific pocket proteins preferentially interact with individual E2Fs. pRB is capable of interacting with E2F1, E2F2, E2F3 and E2F4 while E2F4 and E2F5 interact with p107 and p130. E2F6, E2F7 and E2F8 in contrast do not interact with any pocket proteins (*119*). The E2F transcription factors can also be divided into two distinct functional classes based upon their observed ability to either activate or repress E2F dependent transcription. E2F1, E2F2 and E2F3 are all classified as activator E2Fs due to their ability to strongly activate E2F transcriptional targets while E2F4, E2F5, E2F6, E2F7 and E2F8 are defined as repressor E2Fs due to their ability to block E2F dependent transcription (*120*).

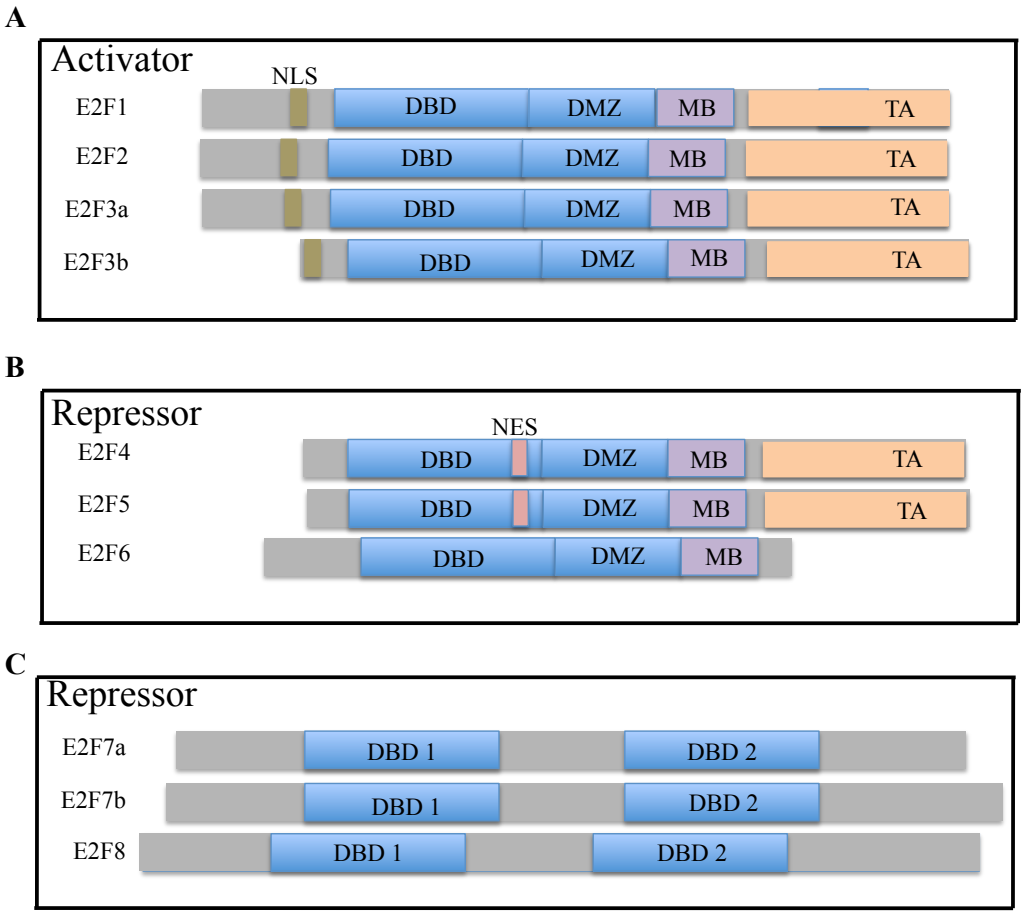
1.5.2 Structural features of the E2F transcription factor

All eight E2F transcription factors share a high degree of similarity in their DNA binding domains (Fig. 1.7). E2F1-6 all contain a dimerization domain that is required for the interaction with DP proteins (*121-123*). The dimerization with DP is critical for the function of E2Fs as E2F1-6 are not capable of interacting with DNA without DP heterodimerization. Both E2F and DP contain a DNA binding domain, that recognizes a E2F recognition sequence through a winged-helix DNA binding motif (*124*). DP proteins share a limited homology with E2F factors, specifically the fold of the DNA binding domain and the sequences that contact DNA are conserved (*124*). While there are three distinct DP proteins (DP1, DP2/3, DP4) the roles of these factors are not well understood and the specificity of the E2F/DP complex is thought to be imparted mainly by the E2F subunit (*119*). E2F7 and E2F8 have two DNA binding domains that allow for the interaction with the E2F recognition sequence independently of DP (*125, 126*).

The recognition sequence in E2F responsive promoters has been characterized largely from *in vitro* experiments (*127*). Further, the consensus sequence was found to differ slightly for individual E2F/DP complexes and for tri-molecular pRB-E2F/DP complexes (*127*). However, *in vivo* studies have suggested that other mechanisms contribute to the specificity of E2F factors as the majority of identified binding sites do not contain a E2F consensus site (*128-130*). E2F1, E2F4 and E2F6 were identified in an overlapping manner at the majority of binding sites *in vivo* suggesting that there is little specificity for individual E2F/DP complexes (*129*). These data suggest that a functional redundancy exists between E2F factors and the association with E2F responsive promoters is mediated by multiple factors that extend beyond a consensus site.

1.5.3 Activator E2Fs

The activator E2Fs, E2F1-3 are highly homologous to one another sharing many of the same domains including the transactivation domain which can activate E2F target gene expression. The C-terminal transactivation domain is capable of recruiting the basal transcription machinery including TFIID as well as co-activating proteins that include, p300, TRAPP and GCN5 to E2F target promoters (*131-134*). It is this domain



Domain structure of the E2F family of transcription factors. The nuclear localization signal (NLS), the nuclear export signal (NES), the DNA binding domain (DBD), the dimerization domain (DMZ), the marked box domain (MB) and the transactivation domain (TA) are shown. (A) The activator class of E2Fs that require dimerization with DP and interact primarily with pRB. (B) The repressor class of E2Fs that also require DP dimerization and do not primarily interact with pRB. (C) The second class of repressor E2Fs that dimerize independently of DP and do not interact with pocket proteins.

Figure 1-7 The E2F family of transcription factors

that pocket proteins interact with and function to block the ability of E2Fs to recruit the transcriptional machinery to E2F target promoters. The activator E2Fs are expressed in a cell cycle dependent manner due in large part to the fact that they are themselves E2F target genes (*135, 136*). Therefore, increased E2F activity creates a positive feedback loop that promotes the expression of more E2F transcription factors. Coupled with the strong nuclear localization signal (*137*) the activating E2Fs represent potent inducers of S-phase target gene expression at the G_1-S transition to drive the cell through the remainder of the cell cycle.

1.5.4 Repressor E2Fs

The repressor class of E2Fs consisting of E2F4 through E2F8 are expressed at constant levels throughout the cell cycle and are thought to primarily act to repress E2F target gene expression. This repressive activity is mediated in part by their lack of a nuclear localization signal that is found on E2F1-3 (*138*). E2F4 and E2F5 also contain a nuclear export signal that limits their accumulation in the nucleus (*139*). The transport of E2F4 and E2F5 to the nucleus requires the formation of complexes with p107 or p130 (*140*). This limits the nuclear pool of E2F4 and E2F5 to E2F-pocket protein complexes which can block proliferation through the recruitment of chromatin remodeling factors to E2F target genes. E2F6-8 do not contain nuclear export signals but lack the transactivation domain (*120*). As such these proteins do not interact with pocket proteins to regulate E2F dependent transcription. E2F6 in contrast interacts with the polycomb repressive complex to mediate the silencing of E2F target genes (*141, 142*). E2F7 and

E2F8 can also function to block transcriptional activation but a co-repressor complex has yet to be defined.

1.5.5 Disruption of E2Fs highlight overlapping and unique roles for E2F family members

A series of gene targeted mouse models have furthered the understanding of the roles of the individual E2F transcription factors. To assess unique roles of individual E2Fs in developmental processes each E2F has been genetically disrupted in the mouse. No one E2F is required for development as all single knockouts are viable in mixed genetic backgrounds. However, each E2F knockout appears to have defects in distinct tissues, which suggests that the individual E2Fs may have tissue specific roles (*120*). Specifically, $E2F4^{-/-}$ mice have craniofacial defects and maturation defects in hematopoietic lineages, which results in increased susceptibility to infections (*143*). $E2F2^{-/-}$ mice were found to develop autoimmune disease due to enhanced T-Cell receptor signaling induced proliferation leading to low concentrations of self-ligand triggering autoimmune reactions (*144*). This suggests that E2F2 may play largely a repressive role through the recruitment of pRB-E2F2 complexes to block the proliferation of naïve T-cells. In contrast, disruption of E2F3 in mice results in reduced viability with a defect in proliferation in cells derived from $E2F3^{-/-}$ mice (*145*). This work has suggested that the individual E2Fs may have tissue specific function and E2F3 may have a larger role in driving the progression into S-phase of the cell cycle.

Recent data has shown that E2F3 is expressed as two distinct isoforms, E2F3a and an N-terminally truncated E2F3b. Gene knockout studies suggest that the two isoforms

have similar functional properties with differences in expression (*146*). A recent study suggests that mammalian development requires only a single activator E2F as disruption of E2F1, E2F2 and E2F3b results in viable mice and disruption of E2F1, E2F2 and E2F3a results in neonatal death at day 19.5 (*147*). Furthermore, E2F1 or E2F3b were found to be sufficient for development if they were expressed from the E2F3a locus (*147*). This suggests that the activator E2Fs are functionally redundant in terms of proliferative control and many of the differences observed may be attributed to differential expression of the E2Fs.

1.5.6 Cycling without E2F transcription factors

Disruption of all activating E2Fs was initially shown to be incompatible with the proliferation of fibroblasts as $E2f1^{-/-}$ $E2f2^{-/-}$ $E2f3^{fl/fl}$ cells infected with Cre recombinase to remove the remaining E2F3 allele, failed to proliferate in culture (*148*). E2F target genes were severely downregulated and these cells failed to enter S-phase. This fits with the critical role for activating E2Fs in the expression of the genes required for S-phase entry. However, more recent experiments have shown that cells are capable of cycling in the absence of activating E2F transcription factors (*149, 150*). Genetic disruption of E2F1, E2F2 and E2F3 results in embryonic lethality by embryonic day 11.5 (*149*). However, at embryonic day 9.5 embryos with disruption of all activating E2Fs can be recovered that appear histologically normal with only subtle defects in proliferation of the myocardium (*149*). The ability of embryos to develop to this stage suggests that E2Fs are not essential for cell division, as a vast amount of division has occurred in the first 9 days of development. An analogous study disrupted all of the E2Fs in the retina of mice and

found that retinal cells could also proliferate in the absence of activating E2Fs (*150*). This proliferation was dependent on the activity of N-myc, which functioned to block the buildup of CDK inhibitors that resulted from the disruption of the activating E2Fs (*150*). These studies suggest that activating E2F activity is not essential for S-phase entry but rather the E2Fs have important contributions to cell cycle advancement that can be compensated by other factors in many cellular contexts.

1.5.7 A unique role for E2F1 in the induction of apoptosis

In many contexts the activating E2Fs have redundant roles in proliferative control. E2F1, however, appears to be uniquely linked to apoptosis. This linkage is highlighted by the fact that loss of E2F1 in mice results in defective apoptosis in thymocytes that leads to a defect in negative selection (*151*). E2f1$^{-/-}$ mice also develop a broad spectrum of tumors between 8 and 18 months of age that includes lymphoma (*152*). This suggests that E2F1 induced apoptosis has a critical role in eliminating pre-neoplastic cells. Importantly, apoptotic defects have not been reported after the disruption of other E2Fs (*144-146, 153-156*), suggesting that this is a unique feature of E2F1.

As described previously *Rb1*$^{-/-}$ animals have a significant increase in apoptosis that was thought to be a direct result of deregulated E2F signaling. In support of this model disruption of E2F1 or E2F3 along in *Rb1*$^{-/-}$ animals attenuates the observed apoptosis and extends the viable development of the embryos (*94, 157*). However, the apoptosis was found to be secondary to a proliferative placental defect that is significantly improved by genetic disruption of *E2f3* (*95*). This suggests that the reduction in apoptosis observed may be a non-cell autonomous event.

Ectopic expression of E2Fs in both cell culture and in transgenic mice has been shown to induce apoptosis (*158-162*). This observation is complicated as the majority of E2Fs are themselves E2F targets and ectopic expression of individual E2Fs results in coincident activation of other E2Fs (*135, 136*). To further investigate this possibility conditional E2F3 transgenic mice were crossed with *E2f1*$^{-/-}$ animals (*163*). Strikingly, E2F3 expression can induce ectopic S-phase entry in the absence of E2F1 but requires E2F1 to actively induce apoptosis (*163*). Thus E2F1 has a unique role in the active induction of apoptosis and other E2Fs appear to drive apoptosis through the activation of E2F1 expression.

Under normal cellular conditions E2F1 does not appear to significantly induce apoptosis, however upon ectopic expression of E2F1 or during DNA damage E2F1 can effectively activate an apoptotic program. E2F1 can induce apoptosis through activation of p53 or its homologue p73 (*164-166*). E2F1 has been shown to activate a series of apoptotic targets including p19-ARF, which functions to inhibit the mdm2-induced degradation of p53 (*167*). E2F1 can also activate p73 directly to induce apoptosis independently of p53 (*168*). The ability of E2F1 to induce apoptosis through p73 is important as the majority of human cancers lack p53 function (*169*). Therefore, the apoptotic response in many tumors is largely mediated by the activity of p73 which is itself primarily activated by E2F1 (*170*). Furthermore, E2F1 can directly activate pro-apoptotic molecules such as Apaf-1 caspases and bid (*171-173*). This suggests that E2F1 has a unique ability to activate both proliferative and apoptotic genes and this may play a functional role in human cancers.

1.5.8 Independent regulation of E2F1 induced proliferation and apoptosis

This dual function of E2F1 raises the question of how E2F1 can under some circumstances, induce proliferation and under other conditions induce apoptosis. One proposed mechanism is the linkage of E2F1 with DNA damage signaling. In response to DNA damage E2F1 undergoes a series of post-translational modifications. E2F1 is phosphorylated by the DNA damage kinases ATM (*174*) and Chk2 (*175*), acetylated by PCAF (*176*) and demethylated by LSD1 (*177*). The modified E2F1 is stabilized and increases the affinity for apoptotic promoters (*168*). In addition pRB is itself modified following DNA damage by Chk1/2 phosphorylation at S612 (*178*), acetylated at K873/874 (*179*) and methylated by Set7/9 and SMYD2 (*180, 181*). The DNA damage modifications of E2F1 and pRB may provide a means to impart a selective induction of E2F1's apoptotic potential. The lack of these modifications under a normal cell cycle could restrict the activity of E2F1 to proliferative promoters. However, the structural basis for the ability of these modifications to direct the activation of E2F1 to specific promoters is poorly understood.

Current understanding of pRB suggests the phosphorylation by CDK complexes in cycling cells results in the release of E2F transcription factors to drive cell proliferation. This has been challenged by work that suggests that E2F1 complexes may persist with phosphorylated pRB under some cellular contexts (*182, 183*). Furthermore, during S-phase in which the majority of pRB is phosphorylated pRB-E2F complexes have been identified bound to specific genomic loci that are not observed in cells in G_1

(*184*). Following DNA damage complexes between phosphorylated pRB and E2F1 have also been identified and correlated with the presence of E2F1-pRB complexes at pro-apoptotic genes including *TP73* (*182*). These complexes are proposed to recruit and activate the histone acetyltransferase PCAF to promote the activation of this gene (*182, 185*). These reports suggest that pRB may be able to maintain the interaction with E2F proteins in its phosphorylated state, however, the mechanism by which phosphorylation can disrupt a subset of E2F complexes but maintain others has yet to be described.

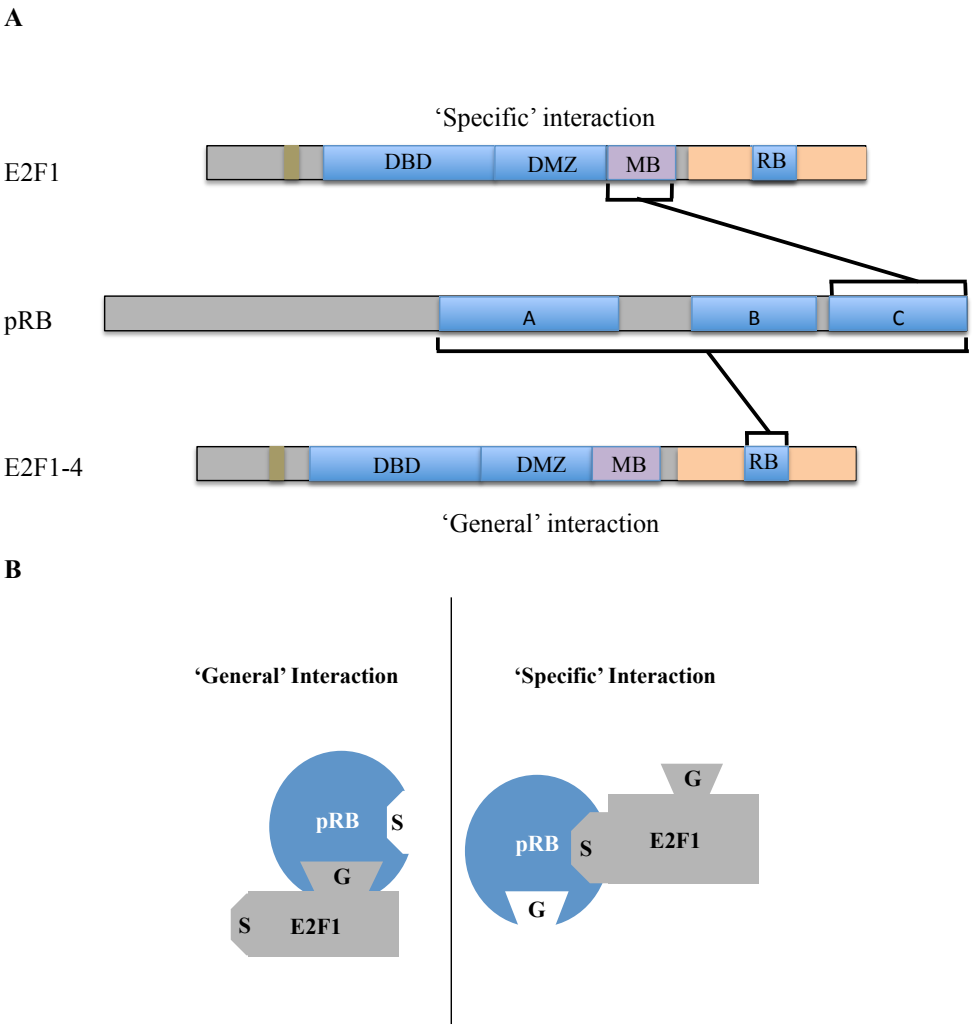
1.6 pRB can regulate E2F1 through two distinct binding sites

1.6.1 Dissecting the distinct E2F binding sites by mutagenesis

In efforts to abrogate E2F binding to pRB it was found that pRB is capable of interacting with E2F1 through two distinct binding conformations (*186*). In addition to the E2F binding site mediated by the large pocket of pRB there is an E2F binding site in the C-terminal region of pRB (*186*). This binding site forms a unique interaction with the marked box domain of E2F1 (*187*). To differentiate between the two binding sites a nomenclature has been developed. The large pocket site that is capable of interacting with E2F1, E2F2, E2F3 and E2F4 is referred to as the 'general site' while the C-terminal E2F1 site is described as the 'specific site' as shown in figure 1.8. As shown in figure 1.8b pRB is capable of forming two distinct interaction types mediated by distinct binding interfaces with E2F1 to form the 'general' or 'specific' complexes. To dissect the function of these individual binding sites synthetic mutants of pRB have been generated

to selectively disrupt the individual interactions (*186-188*). A mutant termed ΔG was generated to disrupt the 'general site' and the ΔS mutant was generated to selectively disrupt the 'specific site' (*186, 187*). The ΔG mutant consists of 11 mutations that span both the A and B halves of the pocket domain. All of the mutations are alanine substitutions that act to disrupt contacts between pRB and E2F complexes across the pocket domain. The large number of mutations required to disrupt E2F binding suggests that the contacts between pRB and E2F extend beyond the binding cleft defined by co-crystallization studies with the E2F transactivation domain (*47, 49*). The ΔS mutation consists of substitutions of M851A and V852A which are found in the C-terminal domain (*187*). These residues are part of the crystal structure recently determined between the C-terminus of pRB with the marked box domain of E2F1 (*50*). These residues are observed to interact with both E2F1 and DP1 in the crystal structure (*50*). The substitutions remove critical contacts at the site to disrupt the 'specific' interaction with E2F1 in isolation.

These mutants have been used to investigate the functional roles of these two distinct binding sites. The 'general site' appears to function to control proliferation while the 'specific site' has been shown to be dispensable for proliferative control (*186-188*). The 'specific site' has been linked to regulation of E2F1 induced apoptosis. Furthermore, forcing the interaction of E2F1 through the 'specific site' in the ΔG mutant results in an increased ability of pRB to block E2F1 induced apoptosis (*186*). Disruption of the 'specific site' in the ΔS mutant in turn blocks the ability of pRB to regulate E2F1 induced apoptosis (*187*). This suggests that the 'specific site' has an active role in E2F1 induced apoptosis. However, the mechanism by which this occurs is not well understood. pRB-



pRB can form two distinct interactions with E2F transcription factors. The C-terminal region of pRB interacts selectively with the marked box domain of E2F1 to mediate the 'specific' interaction. The large pocket of pRB interacts with the transactivation domain of E2F1-4 to mediate the 'general' interaction.

Figure 1-8 pRB contains two distinct E2F binding sites

E2F1 complexes in the ‘specific’ conformation were observed to have low affinity for the canonical E2F DNA recognition sequence (*186*). This suggests that complexes between E2F1 and the pRB in the specific conformation do not function in the traditional manner to repress E2F target genes but rather raises the possibility that they function at a subset of genes and warrants further investigation.

This data suggests that pRB has the ability to regulate the contrasting ability of E2F1 to induce proliferation and apoptosis through the ‘general’ and ‘specific sites’. The mechanisms by which these complexes selectively regulate proliferative or apoptotic targets are not well understood. The E2F1 specific complex is resistant to disruption by E1A as pRB-E2F1 complexes are maintained during adenovirus infection suggesting that the two complexes are regulated through distinct mechanisms (*189*). A recent report has suggested that complexes between pRB-E2F1 can function in response to DNA damage to promote the activation of pro-apoptotic genes (*182*). However, it is not clear which binding site regulates this activity. Other studies have also shown that DNA damage disrupts the interaction with pRB (*190*). To reconcile these contrasting observations there is a need to selectively study the ‘general’ and the ‘specific’ sites in isolation at endogenous conditions to investigate the role of these distinct binding sites.

1.7 E2F independent functions of pRB

1.7.1 E2F independent regulation of differentiation

The current model typically describes the activity of pRB in terms of interaction with E2F transcription factors and the recruitment of chromatin remodeling factors to E2F target genes (*3, 191*). However, data from a synthetic pRB mutant Δ663 that does not interact with E2F or with LXCXE interactors is defective for proliferative control but maintains the ability to regulate differentiation (*192*). This mutant along with other mutations that are defective for E2F binding maintain the ability to activate the expression of muscle or bone specific genes and promote the differentiation of pRB null cells (*192*). Multiple mechanisms have been shown to influence E2F independent differentiation mediated by pRB. In the case of bone differentiation pRB is capable of interacting with and stabilizing complexes between HES1 and RUNX2 which function to activate osteoblast specific genes (*193*). This suggests that pRB can influence the transcription of E2F independent pathways to regulate differentiation.

1.7.2 Regulation of p27 through an E2F independent mechanism by pRB

In addition to the control of differentiation there are reports that suggest pRB can also control proliferation independently of E2F transcription factors. pRB can stabilize p27 through post translational mechanisms that are independent of E2Fs and do not occur in other pocket proteins (*194*). This work was first described in a study published by Ji et al. where expression of pRB in the RB-null osteosarcoma cell line Saos-2 was studied.

When pRB expression was activated in a tetracycline inducible cell line the authors noted that the levels of p27 were upregulated before downregulation of E2F target gene expression occurred (*195*). This further suggested that p27 could be regulated independently of E2F activity. Specifically, pRB was found to regulate the stability of p27 through interacting with the ubiquitin ligase complex containing Skp2 and blocking its ability to target p27 for degradation (*195*). Importantly, mutants that are defective for E2F regulation are still capable of p27 regulation and this has been shown to be a critical aspect of senescence (*194, 195*). pRB is also capable of interacting with components of the anaphase promoting complex (APC) containing the Cdh1 targeting subunit (*196*). Importantly this complex functions in the G_1 phase of the cell cycle to promote the degradation of key proteins including Skp2 to maintain cells in the G_1 phase of the cell cycle. pRB binds to Cdh1 through a LXCXE dependent binding site in the pocket domain and with Skp2 through the C-terminal domain (*195, 196*). By interacting with both of these proteins pRB is capable of promoting the degradation of Skp2. This degradation prevents SCF^{Skp2} complexes from targeting p27 for degradation and results in the stabilization of p27(*196*).

1.7.3 Proliferative control mediated by p27

The critical role for p27 in proliferation is highlighted in *Cdkn1b*$^{-/-}$ animals which display hyperplasia with all organs observed to be at least 20% larger (*112-114*). Loss of p27 results in the development of pituitary tumors in a similar manner to *Rb1*$^{+/-}$ mice (*112-114*). Furthermore, combined disruption of p27 and *Rb1* results in an acceleration of pituitary tumors, a shift to more aggressive tumors and an increase in incidence of thyroid

tumors (*197*). The ability of p27 loss to enhance tumorigenesis of *Rb1*$^{+/-}$ animals suggests that p27 may have pRB independent functions. Notably tumors observed in *Rb1*$^{+/-}$ mice have significant downregulation of p27, which supports the importance of the stabilization of p27 by pRB.

Current models of p27 activity describe a function upstream of pRB to inactivate the CDKs that results in the activation of pRB. Active pRB then is able to repress the transcription of E2F target genes. Importantly, expression of p27 into *RB1* null cell lines induces at least a partial cell cycle arrest (*198*). This in turn suggests that p27 can function downstream of pRB to induce a cell cycle arrest independently of pRB. The mechanism by which this occurs may be dependent upon the inhibition of CDKs by p27. Notably, *Rb1*$^{-/-}$ cells are capable of partially arresting in response to expression of a dominant negative CDK2 protein (*199*). This suggests that down regulation of kinase activity can block the proliferation of cells in the absence of pRB (*199*). As such both pRB and p27 appear to have a partially overlapping role in proliferative control, however, the contribution of these proteins to a given cell cycle arrest is not well described.

1.8 Structure function analysis of the retinoblastoma tumor suppressor protein

1.8.1 A context dependent role for LXCXE interactions

The development of a gene-targeted mouse model that selectively disrupts the interaction with proteins at the LXCXE binding cleft has allowed for a functional role for LXCXE interactions to be defined in multiple cellular contexts (*200*). Fibroblasts

homozygous for this mutation termed ΔL are able to maintain proliferative control in asynchronous growing cultures, in response to serum deprivation and confluence arrest (*200*). Furthermore, the mice are viable and do not develop spontaneous tumors (*58*). However, the cells and mice were found to be defective in their response to other cell cycle arrest stimuli. Specifically, female $Rb1^{\Delta L/\Delta L}$ animals had a reduced ability to nurse their pups resulting in the neonatal lethality of animals raised by $Rb1^{\Delta L/\Delta L}$ mothers (*201*). This correlated with an observed hyperplasia in the ducts of the mammary glands of these animals and an inability of fibroblasts to respond to TGF-β growth arrest (*201*). However, differentiation and cell cycle exit was observed to occur in other developmental contexts in an appropriate manner in the $Rb1^{\Delta L/\Delta L}$ mice (*202*). This work suggested a specific role for LXCXE interactions in cell cycle regulation in a limited set of developmental contexts. Specifically $Rb1^{\Delta L/\Delta L}$ cells treated with TGF-β were found to express elevated levels of E2F target genes suggesting that the recruitment of chromatin remodeling factors was necessary in some cellular contexts to fully repress E2F target gene expression (*201*).

Recent work has highlighted a critical role for pRB in cellular senescence (*107, 202, 203*). Senescence is a permanent form of cell cycle arrest and is thought to have a critical role in blocking the proliferation of pre-malignant cells (*204*). Depletion of pRB in primary human cells induced to undergo senescence results in a failure to appropriately silence the expression of E2F target genes that is required to maintain the senescent state (*203*). This is mediated by the inability of other pocket proteins to localize to these target promoters in the absence of pRB. Furthermore, disruption of the LXCXE binding cleft in $Rb1^{\Delta L/\Delta L}$ fibroblasts also disrupts the ability of pRB to maintain a stable repression of E2F

target genes in senescence (*202*). The LXCXE binding cleft appears to be necessary to sustain the stable repression of E2F target genes to maintain a senescent arrest.

1.8.2 Dissecting the contribution of E2F dependent mechanisms to overall cell cycle arrest

To dissect the contribution of E2F dependent and independent mechanisms to the function of pRB the R661W mutation has been utilized. This mutant is a tumor-derived mutation that is defective for E2F binding. R661 faces the interior of pRB at the interface between the A and B halves of the pocket domain. The substitution of R661W disrupts a hydrogen bond network that abrogates the interface between the A and B regions of the small pocket domain (*39*). This mutant is defective for transcriptional control of E2F but it also disrupts other activities of pRB, as the R661W-pRB is partially defective for LXCXE interactions (*205*). This mutation results in an increase in the interaction with E2F4, which may result from an altered nuclear localization of pRB as E2F4 is normally localized to the cytoplasm (*206*). A gene targeted mouse model has been generated from this mutation by introducing the analogous R654W substitution in the mouse *Rb1* gene. Homozygous mice carrying this mutation are embryonic lethal due to placental defects. However, the placental defects are less severe than in $Rb1^{-/-}$ embryos. This allows for better nutrient transport from the placenta to the embryo proper and the mice typically survive 1-2 days longer than the $Rb1^{-/-}$ mice (*206*). Mice heterozygous for the R654W substitution are also tumor prone with a similar prevalence of pituitary and thyroid tumors as the $Rb1^{+/-}$ mice (*207*). This suggests that E2F binding is required for growth control by pRB. This however, does not fit with the cell culture experiments that have

shown that the R661W mutant when transfected into RB deficient cancer cell lines is capable of inducing an arrest to a similar extent as WT-pRB (*195*). It is possible that the contrasting results are partially mediated by differences in expression of pRB between the endogenous levels in the R654W mice and the levels expressed from the tetracycline inducible cell lines. It is conceivable that the R654W mutation reduces the stability of pRB and alters the nuclear localization such that the levels of R654W-pRB are reduced to levels insufficient to maintain growth control in the absence of E2F binding. Therefore, there is a need to develop new mouse strains that cause defined change in structure that disrupts E2F binding of endogenous pRB without interfering with other binding sites or expression. The generation of this reagent would allow for the study of the functional role of E2F-independent mechanisms of cell cycle control.

1.9 Objectives

As described in this introductory chapter, pRB can utilize multiple mechanisms to regulate proliferation and function as a barrier to tumorigenesis. While pRB has the ability to regulate E2F transcription factors and stabilize p27, the relative importance of these pathways to a given cell cycle arrest is poorly understood. Furthermore, the mechanism by which pRB is capable of independently regulating the proliferative and apoptotic potential of E2F1 has yet to be defined. The overall aim of this thesis is to utilize multiple experimental systems to better define the molecular role of pRB in cell cycle control and E2F1 regulation.

First I examined the structural basis for the existence of E2F1-pRB complexes that were resistant to disruption by CDK phosphorylation. I hypothesized that the

'specific site' may function to regulate the interaction between E2F1 and hyperphosphorylated pRB. Using a cell culture approach in which the phosphorylation state and composition of pRB-E2F complexes could be effectively modulated I characterized the structural basis for these interactions. Further, using site directed mutagenesis the structural basis of the 'specific site' was further defined to elucidate some of the molecular contacts that mediate the unique ability of E2F1 to interact with this region of pRB. These results are discussed in detail in chapter 2 of this thesis.

The complexes identified in chapter 2 between hyperphosphorylated pRB and E2F1 likely exist in cycling cells. Proliferating cultures of primary cells exposed to DNA damage rapidly activate a cellular program that returns pRB to a hypophosphorylated state. While PP1 has been implicated in this process the structural basis for this process was unclear. Our collaborator Dr. Seth Rubin at the University of California, Santa Cruz produced crystallographic and functional data suggesting that the docking site for CDK complexes overlapped with the binding site for PP1. We hypothesized that this shared docking site would produce a competitive binding, in which PP1 could exclude the docking of CDK complexes to hypophosphorylated pRB species and thus block phosphorylation. Using a cell culture approach and *in vitro* binding assays we describe a functional role for the competition between the overlapping PP1 and CDK docking sites in the C-terminal domain of pRB to more rapidly activate it. This work published with the crystallographic and functional data from the Rubin lab (*208*) is presented in appendix 1.

Active pRB can arrest cells in the G_1 phase of the cell cycle. However, the contribution of the distinct interaction surfaces to the overall ability to arrest in G_1 has yet

to be elucidated. To advance our understanding of this process, chapter 3 describes the generation of a series of synthetic mutants that separate distinct elements of cell cycle control mediated by pRB. I hypothesized that each of these binding sites would contribute to the overall ability of pRB regulate proliferative. Using the well-studied ability of ectopic pRB to arrest Saos-2 cells we studied the contribution of these sites in an acute G_1 arrest.

In chapter 3 I suggest that overlapping interaction surfaces function to mediate proliferative control by pRB. As described in chapter 4 I sought to extend this work using a series of mouse models to understand the contribution of the distinct elements of pRB function to cell cycle control. To separate the role of E2F binding from other pathways of cell cycle control I generated a gene-targeted mouse model in which the ability of pRB to regulate E2Fs through the 'general site' was disrupted. I hypothesized that these mice would maintain the ability to control proliferation through other mechanisms that occur independently of the 'general site'. To further investigate these other pathways we crossed our animals with mice lacking p27 and E2F1 to assess the role of p27 stabilization and the 'specific site' in proliferative control and tumorigenesis.

1.10 References

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2 The biochemical basis of CDK phosphorylation-independent regulation of E2F1 by the retinoblastoma protein

2.1 Abstract

The pRB (retinoblastoma protein) has a central role in the control of the G_1–S phase transition of the cell cycle that is mediated in part through the regulation of E2F transcription factors. Upon S-phase entry pRB is phosphorylated extensively, which in turn releases bound E2Fs to drive the expression of the genes required for S-phase progression. In the present study, we demonstrate that E2F1-maintains the ability to interact with ppRB (hyperphosphorylated pRB). This interaction is dependent upon the 'specific' E2F1-binding site located in the C-terminal domain of pRB. A unique region of the marked box domain of E2F1 contacts the 'specific' site to mediate the interaction with ppRB. The mechanistic basis of the interaction between E2F1 and ppRB is subtle. A single substitution between valine and proline residues in the marked box distinguishes E2F1's ability to interact with ppRB from the inability of E2F3 to bind to the 'specific' site in ppRB. The E2F1–pRB interaction at the 'specific' site also maintains the ability to regulate the transcriptional activation of E2F1 target genes. These data reveal a mechanism by which E2F1 regulation by pRB can persist, when pRB is hyperphosphorylated and presumed to be inactive.

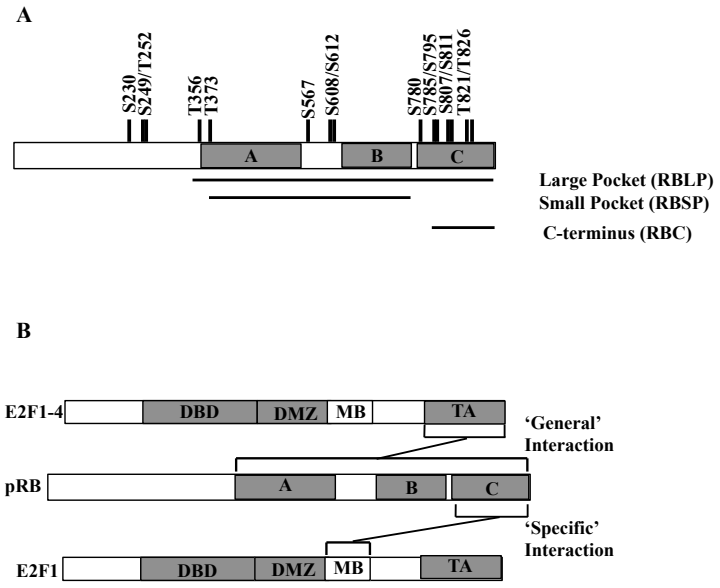
2.2 Introduction

Disruption of the G_1 checkpoint of the cell cycle is a ubiquitous event in essentially all forms of cancer that allows for inappropriate entry into the cell cycle (*1*). The retinoblastoma tumor suppressor protein (pRB) has a central role in the regulation of S-phase entry through its ability to repress the activity of E2Fs (*2*). E2Fs are potent transcription factors that function to activate genes required to progress into S-phase. Mitogenic signaling results in the activation of cyclin-dependent kinase (CDK) complexes, which phosphorylate pRB, and free E2F transcription factors to drive cell cycle progression. The *RB1* gene is mutated only in a small subset of cancers that include retinoblastoma and small cell lung cancer (*3*). Instead, the majority of human cancers express wild-type pRB that exists predominately in an inactive phosphorylated state due to deregulation of CDKs (*4*). Thereby, most human cancers disrupt G_1 checkpoint control upstream of pRB through the deregulation of CDK activity.

Inactivation of pRB by phosphorylation requires the activity of both Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes (*5*). This inactivated form of pRB has often been defined by its slower migration in SDS-PAGE and is commonly referred to as hyperphosphorylated, and abbreviated as 'ppRB'. Hyperphosphorylated pRB has been shown to contain at least 10 distinct phosphopeptides, indicating that it is extensively phosphorylated (*6, 7*), while the faster migrating hypophosphorylated form appears to have limited phosphorylation of some of the same sites (*7, 8*). These observations, combined with mutational analysis of the 16 predicted CDK phosphorylation sites, has lead to a model in which many phosphorylation sites contribute in a redundant manner to

the displacement of E2F binding to ppRB (*9, 10*). The CDK phosphorylation sites are localized to disordered regions of pRB that flank the well-structured pocket domain (Fig. 2.1a) (*11*). The small pocket domain consists of two halves termed A and B (Fig. 2.1a) that each adopt a cyclin-like fold to form a large globular domain that is capable of interacting with E2F transcription factors (*11*). Co-crystallization studies have revealed an interaction between the transactivation domain of E2Fs and the cleft that forms between the two cyclin-like folds of the pocket domain defined as the 'general' interaction (Fig. 2.1b) (*12, 13*). CDK phosphorylation of pRB results in conformational changes of the unstructured regions containing the CDK phosphorylation sites that block the interaction with the transactivation domain of E2Fs (*14*). In this regard, a relatively detailed picture of how phosphorylation regulates pRB-E2F interactions has emerged. However, pRB contains an additional E2F binding site that is utilized exclusively by E2F1 called the 'specific' site (Fig. 2.1b) (*15*). This interaction is mediated by the marked box region of E2F1 and the C-terminus of pRB (Fig. 2.1b) (*16*). The regulatory effects of CDK phosphorylation on this unique pRB-E2F1 interaction are unknown.

There are eight E2F proteins in mammals that share the ability to regulate E2F target genes through a conserved DNA binding domain (*17*). E2F1, E2F2 and E2F3 are defined as activator E2Fs as they contain strong nuclear localization signals and transactivation domains that allow them to induce the expression of S-phase targets (*17*). pRB exclusively regulates the activity of the activator class of E2Fs indicating that they have an intimate relationship in cell cycle control (*18*). Gene-targeting experiments have demonstrated that a single activator E2F can support development in mice, indicating considerable redundancy (*19*). E2F1, however, appears to have a unique role in the



(A) The domain structure of pRB is shown with the large pocket, small pocket, and C-terminal regions delineated. Known CDK phosphorylation sites are shown relative to the domains of pRB. (B) The minimal regions that mediate the 'general' and 'specific' E2F binding interactions are indicated in pRB and E2F.

Figure 2-1 Domain structure of pRB

induction of apoptosis that is distinct from its role in proliferation. The functional significance of this is emphasized by the apoptotic defects that occur in the thymus of E2f1$^{-/-}$ mice and the susceptibility of these mice to multiple tumors including lymphoma (*20, 21*). E2F1 has the unique ability to activate the transcription of pro-apoptotic molecules including ARF, Apaf-1, Caspase 7, Caspase 8, Bid, and p73 (*22-26*). The ability of E2F1 to induce both cell proliferation and cell death necessitates a mechanism by which these contrasting activities can be controlled. Surprisingly, there is little data available to suggest a mechanism by which E2F1-induced apoptosis is controlled independently of proliferation. Using experiments that interchange domains from E2F1 and E2F3, the marked box region of E2F1 has been demonstrated to function in the activation of p73 and p53 induced apoptosis (*27*). The marked box region is a protein-protein interaction domain that has been shown to interact with cellular factors, such as Jab-1 to induce transcription of ARF and apoptosis (*28*), as well as the 'specific' site in the C-terminus of pRB (Fig. 2.1b), which is capable of attenuating E2F1-induced apoptosis (*15, 16*). Based on our current understanding of the regulation of pRB-E2F interactions by CDK phosphorylation at the G_1 to S-phase transition, it is difficult to reconcile how E2F1's pro-apoptotic activity is restrained in normal cells as they enter S-phase and E2Fs are released from pRB's regulation.

Surprisingly, a number of studies suggest the existence of pRB-E2F1 complexes under circumstances where CDK phosphorylation is expected to disrupt their interaction. First, ectopic expression of G_1 Cyclin-CDKs has been shown to have differential effects on E2F1 release from pRB, suggesting that this complex may have altered sensitivity to the kinases relative to other pRB-E2F complexes (*29*). Second, pRB and E2F1 have been

found at the same E2F-responsive promoters and CpG islands in S-phase by chromatin immunoprecipitation (*30*). Third, following DNA damage, pRB-E2F1 complexes have been reported to assemble while at least some phosphorylation sites on pRB remain phosphorylated (*31*). Unfortunately, none of these reports offer a mechanistic explanation that accommodates both the release of E2Fs from hyperphosphorylated pRB and the maintenance of pRB-E2F interactions under the same circumstances.

This study describes a mechanism whereby E2F1 can be bound and regulated by hyperphosphorylated pRB. This interaction is mediated by the C-terminal 'specific' binding site in pRB and the marked box domain of E2F1 (Fig. 2.1b). Despite high conservation of the marked box region of E2Fs, subtle but important sequence differences render only E2F1 capable of interacting with the C-terminus of pRB. Substitution of a single proline to valine in E2F3, to resemble E2F1, is sufficient to create an interaction with phosphorylated pRB. E2F1 interaction with the 'specific' site of pRB is also capable of regulating the activation of a pro-apoptotic gene promoter, whereas this interaction has little ability to regulate other E2F1-dependent transcription. Taken together, this data provides a biochemical basis for the ability of proliferative and apoptotic functions to be differentially regulated by pRB during cell cycle advancement.

2.3 Methods

2.3.1 Plasmid constructions

Site-directed mutagenesis of a pRB cDNA was carried out by PCR as previously described (*32, 33*). Mutants were introduced into the bacterial GST-RBLP (Large Pocket

Domain) or the GST-RBC (C-terminal domain only) cloned into the pscodon vector (Delphi Genetics). Mutagenesis of E2Fs was carried out in a similar manner using an E2F cDNA cloned into pBluescript and later subcloned into the CMV-HA expression vector. All subclones of PCR products were sequenced to ensure that they only contained the desired mutations. CMV-HA-E2F1, -E2F2, E2F3, -DP1, and their sources have been described previously (*15*). CMV-CDK2, -CDK4 and -DN-CDK2, were reported initially by van den Heuvel and Harlow, 1993 (*34*). CMV-Cyclin D and –Cyclin E were reported initially by Hinds *et al.,* 1992 (*35*). p73-Luc plasmid was reported initially by Urist *et al.,* 2004 (*36*). The myc-PP1 construct was reported by Traweger *et al.,* 2008 (*37*).

2.3.2 Cell Culture

C33A and T98G cell lines were obtained from ATCC and cultured according to standard methods. Cell culture was carried out in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2mM L-glutamine, penicillin (50U/mL) and streptomycin (50μg/mL). The C33A cells were used to generate extracts for GST-pull-down and co-transfection immunoprecipitation experiments. The C33A cells were transfected using calcium phosphate with the precipitates left on the cells for 16h before fresh growth medium was added. The T98G cells were used to characterize endogenous complexes between pRB and E2Fs synchronized by serum starvation for 72 hours in media with 0.1% fetal bovine serum. The cells were stimulated to re-enter the cell cycle with media containing 20% fetal bovine serum.

2.3.3 Immunoprecipitations and Western blotting

GST-pull-down and co-immunoprecipitation assays were performed as previously described (*33*). To generate extracts for these experiments C33A cells were plated at a density of 6 X 10^6 cells in a 15cm dishes and transfected with a total of 60μg of DNA using calcium phosphate. 48 hours after transfection the cells were harvested. To generate extracts for GST pull-down assays the cells were washed twice with phosphate buffered saline (PBS) and collected into 1mL of Gel Shift Extract (GSE) buffer (20mM Tris pH 7.5, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, 5μg/mL leupeptin, 5μg/mL aprotinin, 0.1mM Na_3VO_4, 0.5mM NaF and 1mM DTT). Extracts were frozen at -80°C. To generate extracts for co-immunoprecipitation of pRB-E2F complexes nuclear extracts were prepared. Briefly, cells were washed twice and collected in 1mL of PBS. Cells were resuspended in three times the cell volume of hypotonic lysis buffer (20mM Tris pH 7.5, 10mM KCl, 3mM $MgCl_2$, 1mM EDTA, 1mM PMSF, 1mM DTT, 5μg/mL leupeptin, 5μg/mL aprotinin, 5mM NaF, 0.1mM Na_3VO_4 1mM DTT). Extracts were incubated on ice for 5 min before 0.05% NP-40 was added to the hypotonic lysis buffer and the extracts were incubated on ice for a further 5 min. Nuclei were pelleted by centrifugation at 4°C at 4000rpm for 6 min and washed two times with hypotonic lysis buffer containing 0.05% NP40. Nuclei were resuspended in GSE buffer and frozen at -80°C

Extracts were thawed and cellular debris was removed by centrifugation at 14,000 rpm. For co-transfection immunoprecipitations C33A extract was diluted in IP wash buffer (20mM Tris pH 7.5, 200mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25mM DTT

and 0.1% NP-40). pRB complexes were immunoprecipitated with 12CA5 for HA-tagged E2Fs, C-20 (Santa Cruz) for E2F1 and C-18 (Santa Cruz) for E2F3, bound to protein G-sepharose (GE healthcare). Immunoprecipitations were incubated with rocking for 1 hour at 4°C. The protein G-sepharose beads were washed twice with IP wash buffer then eluted in 1X-SDS-PAGE sample buffer and resolved by electrophoresis on a sodium dodecyl sulfate 8% polyacrylamide gel electrophoresis (SDS-8% PAGE) gel. Proteins were transferred to a nitrocellulose membrane by standard techniques. HA-tagged E2Fs were detected using 3F10 (Roche), E2F1 by KH20 (Santa Cruz), E2F3 by PG37 (Upstate), Myc-tagged PP1 by 9E10 and pRB by G3-245 (BD Pharmingen).

2.3.4 GST pulldown binding experiments

GST-fusion proteins were expressed in BL21-DE3-Gold *E.coli* (Stratagene) in 500mL cultures. Briefly, cells were grown for 2 hours at room temperature after which 100μM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures and the cultures were grown overnight at 16°C. The following morning the cells were harvested and GST-fusion proteins were purified using glutathione sepharose according to standard protocols. Purified GST-fusion protein (2μg) was diluted in low salt GSE buffer (20mM Tris pH 7.5, 200mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 1mM DTT and 0.1% NP-40) and incubated with 100mL of whole cell C33A extract expressing HA-E2Fs or myc-PP1. GST-pRB complexes were precipitated with glutathione sepharose and washed twice with low salt GSE buffer and eluted with 1X-SDS-PAGE sample buffer. Samples were electrophoresed on an SDS-8% PAGE gel and blotted using the same antibodies outlined for the immunoprecipitation experiments.

2.3.5 Luciferase reporter assays

Transcriptional reporter assays were carried out as reported previously (*33*). Saos-2 cells were plated in 6 well plates at a density of 5 X 10^5 cells per well. Cells were transfected with 100ng of the E2F4B-luciferase reporter or 200ng of the p73-Luc reporter plasmid along with 15ng of CMV-HA-E2F, 15ng CMV-HA-DP1 and 200ng of CMV-β-Gal. Increasing concentrations of CMV-pRB expression plasmid were transfected to block the activity of the transfected E2Fs. Total plasmid DNA was normalized with the addition of CMV-CD20. Cells were harvested 36 hours after transfection with 1X Reporter Lysis Buffer (Promega). The luciferase activity was determined with the Luciferase assay system (Promega) and normalized to β-gal activity. The β-gal activity was determined using standard techniques to measure the hydrolysis of 2-Nitrophenyl-β--D-galactopyranoside (ONPG) at 405nm. The average of three independent transfections is shown and the error bars indicate one standard deviation from the mean.

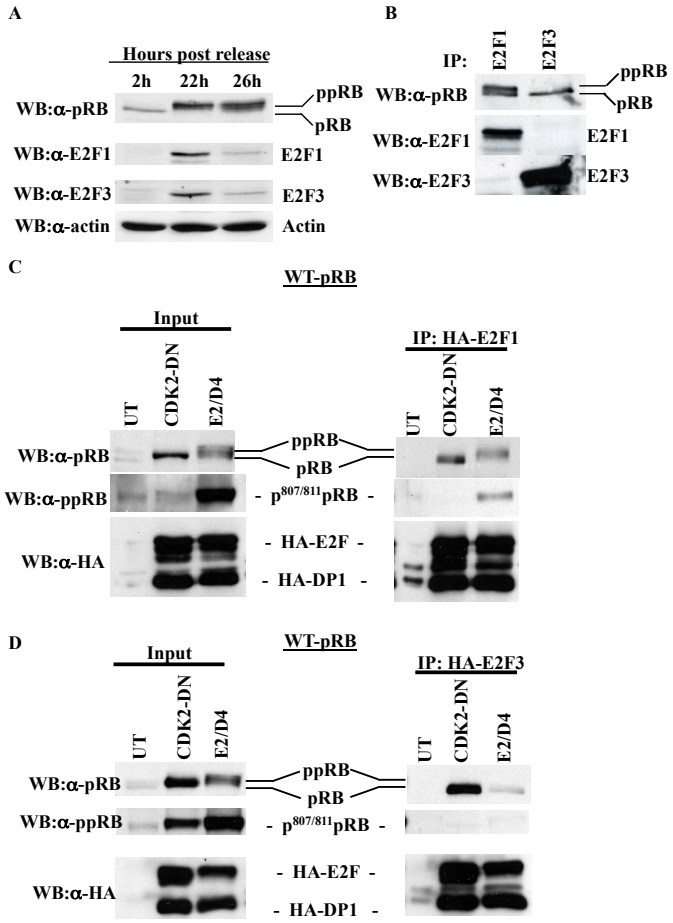
2.4 Results

2.4.1 A unique interaction between E2F1 and hyperphosphorylated pRB

The disruption of pRB-E2F complexes by phosphorylation is thought to be a critical event of the G_1-S phase transition of the cell cycle. Gel shift experiments have described the release of E2Fs from pRB upon S-phase entry (*38, 39*). These experiments

have often utilized a double-stranded DNA probes from the adenovirus E2 promoter that contain a canonical E2F site and is bound by E2Fs and pRB-E2F complexes (*38, 39*). Complexes formed between pRB and E2F1 using the C-terminal 'specific' site have low affinity for this type of probe (*15*), and as such pRB-E2F1 'specific' complexes are not observed in gel shift experiments. Thereby, previous work that has described the release of E2Fs following cell cycle entry only pertains to the 'general' E2F interaction that is common among E2Fs that interact with pRB. To explore other binding sites that may be regulated independently of phosphorylation at the G_1-S transition we utilized co-immunoprecipitation to directly assess the ability of E2Fs to interact with all binding sites in ppRB.

T98G cells, which have an intact G_1 checkpoint (*40*), were synchronized by serum starvation for 72 hours, then induced to re-enter the cell cycle with media containing 20% serum. Initially, pRB exists primarily in a hypophosphorylated state and further culture of cells in high serum results in a significant enrichment for ppRB (Fig 2.2a). Extracts from cells synchronized to enrich for ppRB were immunoprecipitated with E2F1 and E2F3 antibodies. Both E2F1 and E2F3 are capable of interacting with and co-immunoprecipitating pRB (Fig 2.2b). E2F3 only interacts with the hypophosphorylated form of pRB (Fig. 2.2b) suggesting that phosphorylation disrupts the interaction between ppRB and E2F3. In contrast, E2F1 can immunoprecipitate both pRB and ppRB as determined by the electrophoretic mobility of the precipitated proteins (Fig 2.2b). The use of the shift in apparent molecular weight to detect ppRB ensures that it is extensively phosphorylated. This provides experimental evidence for complexes between



T98G cells were synchronized by serum starvation followed by stimulation with media containing 20% FBS. (A) Expression of pRB and E2Fs following addition of high serum media was determined by western blotting. Actin was used as a control for protein loaded. (B) Immunoprecipitation of E2F-pRB complexes with E2F1 or E2F3 specific antibodies from T98G cells 26 hours after addition of serum. Bound E2F1, E2F3 and pRB protein was detected by western blotting. (C, D) C33A cells were co-transfected with expression plasmids for pRB and HA-tagged forms of E2F1, or E2F3, and DP1. The phosphorylation status of pRB was modulated by transfection of CDK2-DN to produce hypophosphorylated pRB or transfection of Cyclin D/ CDK4 and Cyclin E/CDK2 (denoted D4 and E2 respectively) to produce hyperphosphorylated ppRB. UT denotes untransfected extracts. Western blots of input controls are shown in the left most panels. The interaction of E2F1 or E2F3 with pRB and ppRB was detected by western blotting of immunoprecipitates for different phosphorylation states of pRB as determined by electrophoretic mobility shift and reactivity with phospho-specific antibodies.

Figure 2-2 E2F1 forms a unique complex with ppRB in T98G cells

endogenous ppRB and E2F1 and suggests there is functional relevance to E2F1 regulation after S-phase entry.

To further characterize the interaction between E2Fs and ppRB, the *RB1*-null cell line C33A was utilized. These cells do arrest in response to the ectopic re-expression of pRB, which allows for its phosphorylation state to be modulated independently of the cell cycle phase. To produce hypophosphorylated pRB, a dominant negative CDK2 (CDK2-DN) was expressed to block the activity of endogenous CDK complexes. Alternatively, hyperphosphorylated ppRB was produced by expression of CyclinD/CDK4 and CyclinE/CDK2 complexes (denoted as E2/D4 in Fig. 2.2c). As shown in figure 2.2c, modulation of the kinase activity is sufficient to shift the ectopically expressed pRB from a hypophosphorylated to hyperphosphorylated state as determined by the electrophoretic mobility shift, as well as with phospho-specific antibodies raised against phosphorylated S807/S811. HA-tagged E2F and DP1 constructs were co-transfected and immunoprecipitated with a HA-specific antibody. The use of a HA-antibody excludes potential differences in the E2F1 and E2F3 antibodies to recognize E2F-ppRB complexes. This provides a system in which the phosphorylation state of pRB can be modulated to investigate the interaction of ppRB with different E2F transcription factors.

In cells expressing CDK2-DN, both HA-E2F1 and HA-E2F3 are capable of interacting with pRB, confirming that either can immunoprecipitate pRB in its hypophosphorylated state (Fig 2.2c,d.). In cells expressing CDKs to produce predominately ppRB, HA-E2F3 is only capable of interacting with and immunoprecipitating the small amount of residual hypophosphorylated pRB that remains (Fig. 2.2d). In contrast, HA-E2F1 is capable of immunoprecipitating ppRB as determined

by both the electrophoretic mobility shift and with phospho-specific antibodies shown in figure 2.2c. This suggests that E2F1 has a significant affinity for ppRB that allows for the formation of stable complexes between these proteins. In contrast, hyperphosphorylation of pRB is sufficient to abrogate the binding of E2F3, revealing differential regulation of E2F1 and E2F3 by pRB.

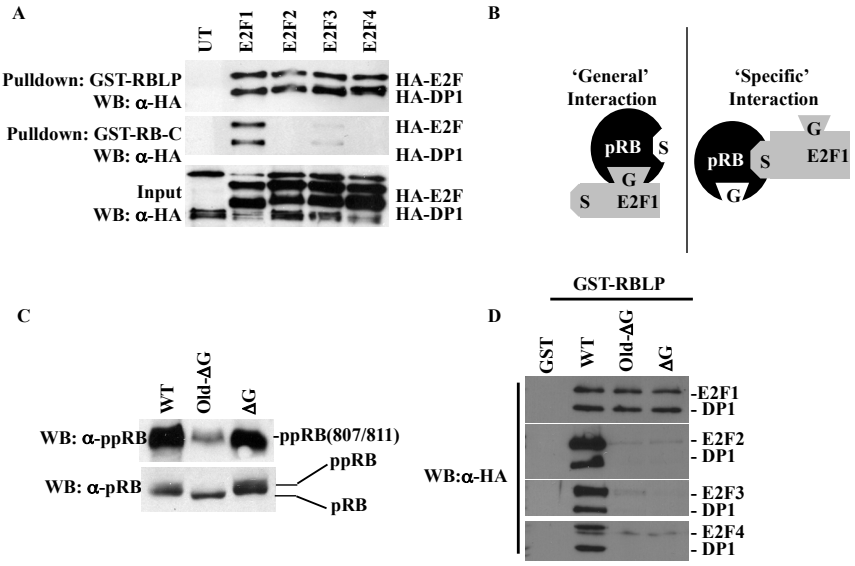
Our experiments demonstrate that complexes between ppRB-E2F1 can be detected at endogenous levels, suggesting that this interaction occurs as part of normal cell cycle progression. While both E2F1 and E2F3 are capable of efficiently interacting with hypophosphorylated pRB, only E2F1 is capable of forming an interaction with hyperphosphorylated pRB. This demonstrates that the interaction with ppRB is a unique feature of E2F1 that allows for its independent regulation.

2.4.2 The 'specific' interaction is a unique feature of pRB and E2F1 that mediates the E2F1-ppRB complex

Detailed reports have described the structural mechanisms by which phosphorylation of pRB results in the release of bound E2F transcription factors. These studies report that phosphorylation induces multiple conformational changes in pRB that function to abrogate interaction with E2Fs (*10, 14, 41, 42*). The interaction between ppRB and E2F1 observed in this study is an apparent contradiction to the structural models of pRB phosphorylation. However, many of these previous studies predate the identification of the 'specific' E2F1 binding site found in the C-terminus of pRB (Fig. 2.1b). Therefore, we next sought to determine if the 'specific' site mediates the interaction between ppRB and E2F1.

The two distinct E2F binding sites on pRB can be studied through the use of recombinant proteins that contain the entire large pocket of pRB (RBLP) or the C-terminus (RB-C). Recombinant proteins were incubated with extract from C33A cells expressing HA-E2F and HA-DP1 proteins. As shown in figure 2.3a RBLP is capable of precipitating complexes with HA-E2F1, HA-E2F2, HA-E2F3 and HA-E2F4. In contrast, RB-C only precipitated HA-E2F1 in appreciable amounts (Fig. 2.3a.). Taken together this confirms that RBLP contains both the 'general' and the 'specific' E2F binding sites while RB-C essentially contains only the 'specific' site. Furthermore, the availability of two distinct E2F1 binding sites allows E2F1 to adopt both interaction types interchangeably as shown in figure 2.3b. However, the mechanism by which E2F1 contacts the 'specific' site has not been extensively studied and raises the possibility that it may mediate the observed interaction between ppRB and E2F1.

To assess the interaction of E2F1 at the 'general' and 'specific' binding sites of ppRB, mutants of pRB were utilized that disrupt the individual E2F binding sites in isolation. Previously a mutant termed ΔG was reported to disrupt the interaction between pRB and E2Fs at the 'general' site (*15*). This mutant contains substitutions in the A, B and C-terminal regions, including K873 and K874, which are important contact sites of CDK2 associated cyclins and is necessary for efficient phosphorylation (*43*). As a result, this mutant, defined as Old-ΔG for this study, is not phosphorylated to the same extent as the WT protein (Fig. 2.3c). For this reason a new ΔG mutant was created that selectively disrupts the 'general' E2F binding site while other binding sites remain intact. This mutant contains R467E and K548E substitutions and is phosphorylated to a similar extent as WT-pRB when transfected into C33A cells (Fig. 2.3c). In a similar manner to the old-

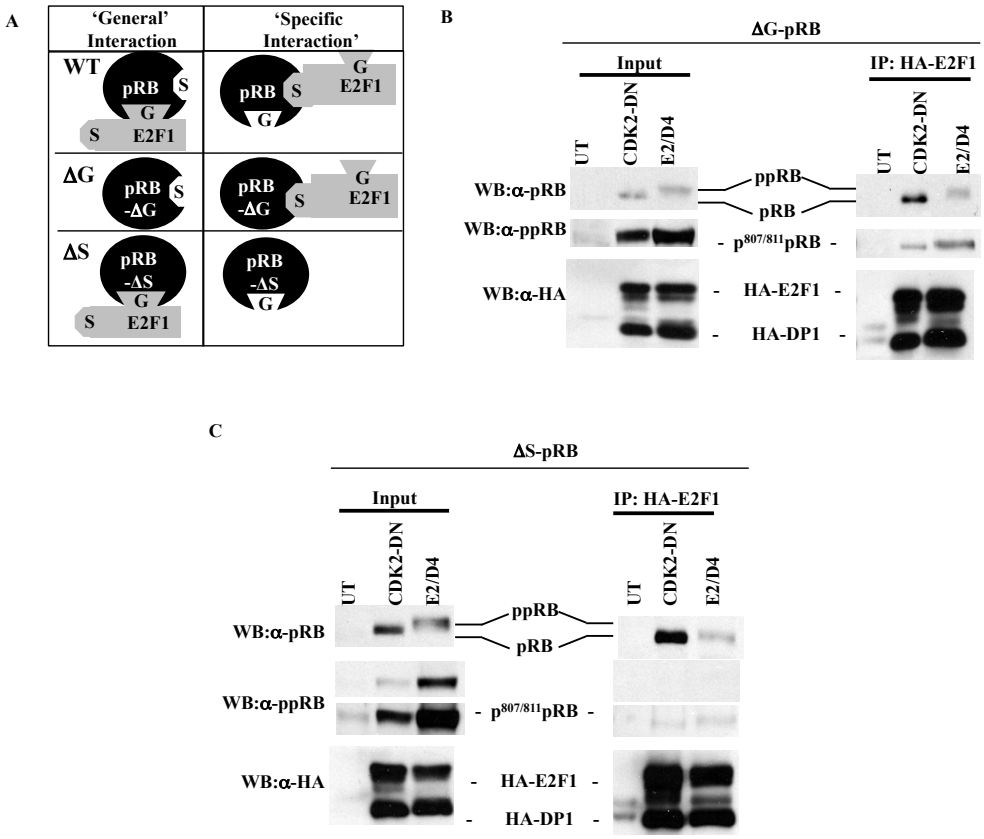


(A) Recombinant GST-RBLP and GST-RB-C proteins were incubated with extracts containing HA-E2Fs and precipitated using glutathione sepharose. The relative amount of input (bottom panel) and bound HA-E2Fs (top panels) were detected by western blotting. (B) Schematic diagram outlining the ability of E2F1 to interact with pRB through two distinct interaction surfaces at either the 'general' or 'specific' sites. (C) C33A cells were transfected with pRB mutants, and endogenous Cyclin/CDK phosphorylation was detected by western blot. Phosphorylation was assessed using differences in electrophoretic mobility shift and the reactivity with a phospho-specific RB antibody against S807/811. (D) Recombinant GST-RBLP mutants were incubated with extracts transfected with HA-E2Fs and HA-DP1. The amount of bound HA-tagged E2F was determined by western blotting.

Figure 2-3 Differentiating between 'specific' and 'general' E2F interactions with pRB

ΔG mutant, the new ΔG mutant disrupts the ability of E2Fs to bind to the 'general' site but maintains the ability to interact with E2F1 through the 'specific' site. This is highlighted by the inability of recombinant ΔG-pRB to interact with HA-E2F2, HA-E2F3 or HA-E2F4 but maintain an interaction with HA-E2F1 (Fig. 2.3d).

As depicted in figure 2.4a the novel ΔG-pRB mutant and the previously reported ΔS mutant (*16*) provide a means to study the two distinct E2F binding sites. The ΔG mutant selectively disrupts the 'general' site in order to study the 'specific' site, while the ΔS mutant disrupts the 'specific' site allowing the 'general' site to be studied in isolation. This allows us to determine the binding site that mediates the observed complex between ppRB and E2F1. In a similar manner to our previous experiments, C33A cells were transfected with combinations of CDK complexes to modulate the phosphorylation state of the ΔG and ΔS pRB mutants. As shown in figure 2.4b and c both the ΔG and ΔS pRB proteins are extensively phosphorylated by expression of CDK complexes. As shown in figure 2.4b, E2F1 is capable of immunoprecipitating both the ΔG-pRB and ΔG-ppRB species, suggesting that the 'specific' site is sufficient to mediate the observed ppRB-E2F1 complex. To investigate this further the ΔS mutation was employed to selectively disrupt the 'specific' site, thus directing E2F1 to the 'general' site. As is shown in figure 2.4c, the ΔS mutant is also extensively phosphorylated when CDKs are expressed, however, HA-E2F1 is only capable of interacting with and immunoprecipitating the hypophosphorylated ΔS-pRB. The small amount of residual pRB that is precipitated migrates at the hypophosphorylated size and has almost no detectable phosphorylation at



(A) Schematic outlining the ability of the ΔG and ΔS mutants to selectively block the 'general' or 'specific' sites in order to study distinct E2F interactions in isolation. (B,C) pRB mutants and HA-E2Fs were transfected into C33A cells and the phosphorylation state was modulated by the expression of DN-CDK2 or active Cyclin E/CDK2 and Cyclin D/CDK4 (designated E2/D4). Input levels of transfected proteins are shown in the left most panels. pRB and ppRB were co-immunoprecipitated with HA-E2F1 and detected by western blotting. The phosphorylation state of pRB was determined by the electrophoretic mobility shift and with phospho-specific antibodies raised against phosphorylated S807/S811.

Figure 2-4 The 'specific site' is required for the interaction between E2F1 and hyperphosphorylated pRB

S807/S811 (Fig. 2.4c). This reveals a critical role for the 'specific' site in ppRB-E2F1 complex formation.

This provides a biochemical basis for the observed ability of E2F1 to maintain an interaction with ppRB while other E2Fs are released. The 'specific' site is necessary and sufficient to mediate the interaction with ppRB. Furthermore, it reveals that the 'general' and 'specific' sites are regulated independently from one another, as the 'general' binding site is regulated by CDK phosphorylation while the 'specific' binding site is resistant. Therefore, the 'specific' site provides a means for regulating E2F1 independently of cell cycle position.

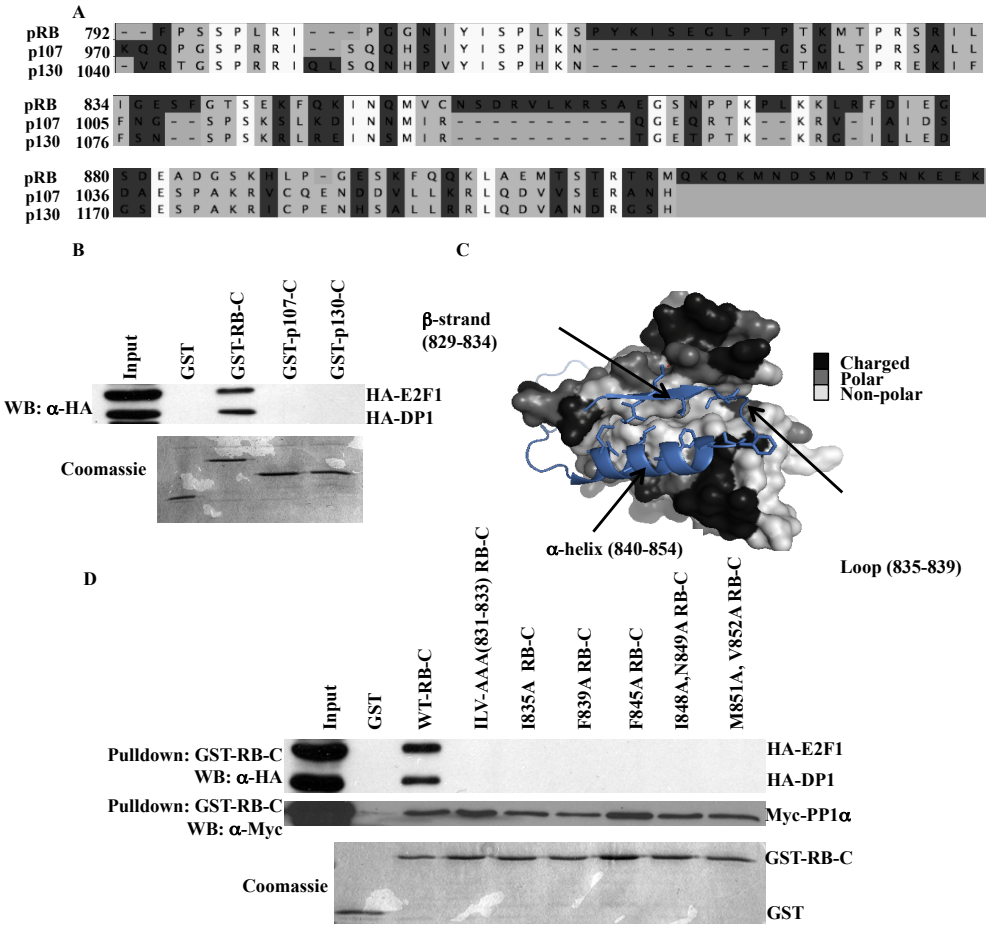
2.4.3 Unique structural elements of pRB and E2F1 mediate the 'specific' interaction and ppRB-E2F1 complexes

The 'specific' site has been previously localized to the C-terminal domain of pRB and the marked box domain of E2F1 (*16*), however, there is still little understanding of the structural basis for E2F1's unique interaction with pRB at this site. The critical role of the 'specific' site in mediating the complex between hyperphosphorylated pRB and E2F1 underscores the importance of understanding the structural basis for the interaction between the C-terminus of pRB and E2F1 and motivated us to investigate it in more detail.

pRB is a member of a family of proteins termed the pocket proteins, which share a well conserved pocket domain. Interestingly, while the C-terminal region contributes to E2F binding by all pocket proteins, there is little conservation between the C-terminal

domains of pRB and its other family members (Fig. 2.5a). Furthermore, p107 and p130 share common sequence elements that are distinct from pRB suggesting that this region differentiates pRB from its two closest relatives (Fig. 2.5a). To investigate the role of the C-terminus of pocket proteins in E2F1 binding, we generated GST-C-terminal constructs of p107 and p130 termed p107-C and p130-C. These constructs consist of the polypeptides that are aligned in figure 2.5a. They are sequences from p107 and p130 that begin immediately C-terminal to the small pocket domain and extend to the C-terminus. These recombinant proteins were incubated with extracts containing HA-E2F1 and HA-DP1, and complexes were precipitated with glutathione sepharose. Only GST-RB-C is capable of precipitating HA-E2F1 complexes as shown in figure 2.5b. This suggests that the 'specific' site is a unique feature of pRB that differentiates its interaction with E2F1 from other pocket protein-E2F1 interactions.

Crystallographic data has described the interaction between a fragment of the C-terminus of pRB and the marked box domain of E2F1 and DP1 (*42*). Since these regions have previously been mapped as the site of interaction for the 'specific' site (*16*), we designed experiments to investigate if its structural features contribute to the 'specific' E2F1-pRB interaction. This crystal structure shows how a small pRB fragment interacts with a hydrophobic cleft that is formed by the marked box domain of E2F1 and DP1 (Fig. 2.5c). We used a computational alanine scanning mutagenesis approach to identify critical interaction sites between pRB and E2F1. The residues identified were largely hydrophobic and include I831, L832, V833, I835, F839, F845, I848, N849, M851 and V852 of pRB. Most of these amino acids form contacts found on the α-helix, the loop,

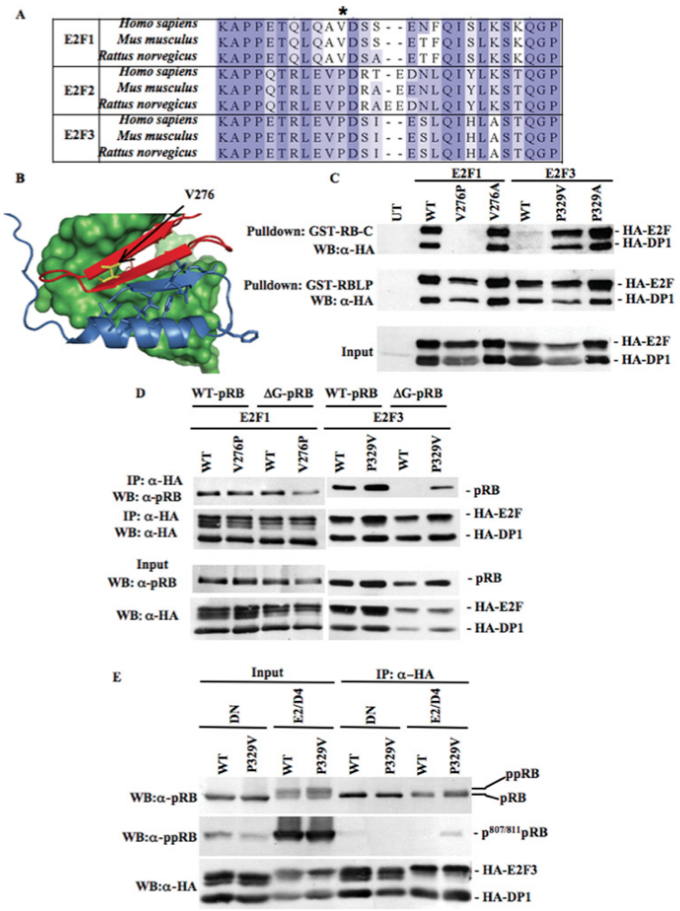


(A) The 'specific' site is a unique feature of RB-C. The C-terminus of the other pocket proteins, p107 and p130 was determined as the terminal region that extended past the conserved small pocket. The C-termini of pRB (NP_000312) p107 (NP_002886) and p130 (NP_005602) were aligned using ClustalW and shaded based on their conservation. Dark shading indicates complete conservation of a specific residue, light shading represents conservative changes and no shading denotes the absence of conservation. (B) Recombinant GST fusion proteins with the C-terminal regions of pRB, p107 and p130 were incubated with extracts expressing HA-E2F1. Complexes were precipitated with glutathione sepharose and bound HA-E2F1 was detected by western blotting. Input levels of the recombinant proteins are shown in the coomassie stained gel. (C) Hydrophobic contacts of RB-C interact with a hydrophobic cleft formed by E2F1/DP1 heterodimers. The amino acid side chains that contact RB-C are colored in blue. E2F1/DP1 are shaded based on their hydrophobicity. Crystal coordinates are from PDB:2AZE. (D) Recombinant GST-RB-C proteins were generated in which the indicated hydrophobic amino acids were changed to alanine. These proteins were incubated with extracts expressing HA-E2F1/HA-DP1 or Myc-tagged PP1 and precipitated using glutathione sepharose. The amount of bound HA-E2F1/HA-DP1 or Myc-PP1 was detected by western blotting and the input levels of the recombinant RB-C proteins were determined by coomassie staining.

Figure 2-5 Characterization of critical structural contacts that define the 'specific site'

and β-strand portions of pRB in this structure (Fig. 2.5c). They contact a patch of hydrophobic residues at the E2F1-DP1 interface (Fig. 2.5c). To test the importance of these residues in maintaining the interaction between pRB and the marked box domain of E2F1, RB-C constructs were generated with substitution of the predicted contacts to alanine. As is shown in figure 2.5d, these amino acids appear to have a critical role in maintaining the interaction with E2F1 as mutation of any of them is sufficient to disrupt interaction with E2F1/DP1 in GST-pulldown experiments. To ensure the integrity of the RB-C proteins, their interaction with Myc-PP1 was characterized. The recombinant RB-C mutants were incubated with PP1 which is known to interact with this region of pRB (*44*), and the ability of the mutants to precipitate PP1 suggests that these are specifically defective for binding to E2F1 (Fig. 2.5d). This indicates that these residues are essential components of the 'specific' E2F1 binding site in the C-terminus of pRB.

The 'specific' site of pRB interacts with a region of E2F1 known as the marked box. This region is the site of multiple protein-protein interactions and is well conserved between distinct E2F family members (Fig. 2.6a). The conservation of this region does not correlate with the selective interaction between the marked box domain of E2F1 and the 'specific' site. This region, shown in figure 2.6a, is largely conserved between E2F1, E2F2, and E2F3 with a few exceptions. Of particular interest is V276 (denoted by *) in E2F1. The valine at position 276 of E2F1 is conserved in closely related mammals such as human, mice and rats but in E2F2 and 3 this residue is strictly conserved as a proline (Fig. 2.6a). V276 localizes to the distal end of the β-sheet of E2F1 that is in closest



(A) Alignment of E2F1, E2F2, and E2F3 proteins using ClustalW and shaded to reflect the conservation with darkly shaded blocks indicating complete conservation of a specific residue, light shaded blocks indicated partial conservation, and no shading represents residues that have non-conserved substitutions. V276 of E2F1 is denoted (*). (B) Crystal structure of RB-C (Blue) bound to E2F1 (Red) and DP1 (Green) with V276 of E2F1 highlighted in yellow (PDB: 2AZE). (C) HA-E2F1 mutants V276P and V276A along with HA-E2F3 mutants P329V and P329A were transfected into C33A cells and incubated with recombinant GST-RBLP or GST-RB-C. Proteins precipitated with glutathione sepharose were detected by western blotting (upper panels). Input levels of the transfected proteins are shown in the bottom panel. (D) C33A cells were co-transfected with expression plasmids for pRB and HA-tagged forms of E2F1 and E2F3 mutants, along with DP1. The lower western blots represent the input expression levels of the proteins in these extracts. The amount of pRB that co-precipitates with HA-E2F is shown in the upper panels. (E) The phosphorylation status of pRB was modulated using either expression of DN-CDK2 to produce hypophosphorylated pRB or expression of CDK complexes to produce hyperphosphorylated ppRB. Input expression levels of E2F and pRB proteins are shown on the left. The interaction of WT-E2F3 and P329V-E2F3 with ppRB was determined using western blotting to detect the electrophoretic mobility shift of ppRB and with phosphorylation specific antibodies.

Figure 2-6 Identification of a structural element in E2F1 that defines selectivity for the 'specific site'

proximity to the co-crystallized pRB fragment shown in figure 2.6b. The marked box domain in E2F2 or E2F3, which contains the conformationally restricted proline at this site, likely adopts a distinct structural conformation compared to E2F1 in this region. For this reason we expect it to be incompatible for interacting with the 'specific' site of pRB as depicted by this crystal structure.

To investigate the effects of a V276P substitution on the interaction with the 'specific' site of pRB, an HA-tagged E2F1-V276P was expressed in C33A cells. Figure 2.6c, shows that the V276P mutant maintains the interaction with RBLP, presumably through the 'general' site. However, this mutant is unable to interact with RB-C, which measures interactions at the 'specific' site. This suggests that the substitution does not disrupt the overall fold of E2F1 or the interaction with DP1 but selectively disrupts the interaction with the 'specific' site of pRB. Furthermore, substitution of V276A does not disrupt the interaction with RB-C, suggesting an important role for proline in determining compatibility for binding to the 'specific' site. The V276P-E2F1 mutant was transfected into C33A cells along with full-length WT-pRB or ΔG-pRB to further characterize the interaction of V276P-E2F1 with the 'general' and 'specific' binding sites. As shown in figure 2.6d the V276P substitution does not disrupt the interaction with WT-pRB suggesting that the overall integrity of E2F1 is maintained. The V276P substitution, however, leads to a partial disruption in the interaction with ΔG-pRB, which only contains the 'specific site' (Fig. 2.6d). The remaining binding to full length ΔG-pRB is likely mediated by contact sites or structural features that exist outside of the C-terminal domain of pRB. Taken together this suggests that the introduction of the proline in the marked box domain of E2F1 is sufficient to disrupt the interaction with the 'specific site'.

As shown previously, E2F3 is unable to interact with the 'specific' site found in the C-terminal domain of pRB (Fig. 2.3a). Strikingly, substitution of the analogous proline (P329) to a valine in E2F3 results in a gain of interaction with RB-C (Fig. 2.6c). Substitution of P329 to alanine in E2F3 is also sufficient to allow E2F3 to interact with RB-C. This suggests that the inability of E2F3 to interact with the 'specific' site is due in part to P329 and its effect on this region of the marked box domain. To further validate the ability of the proline in the marked box domain of E2Fs to prevent interactions with the 'specific' site, the ΔG mutant was employed to selectively disrupt the 'general' site in order to study the interaction with the 'specific' site in isolation. As is shown in figure 2.6d, transfected HA-E2F3 is unable to interact with ΔG-pRB as it is unable to bind to the 'specific' site. Once again, substitution of P329V is sufficient to mediate the interaction with the 'specific' site of pRB, as the mutant protein is able to interact with both WT and ΔG-pRB. This further confirms that the presence of a proline in the marked box domain creates a distinct conformation in E2F3 that prevents the interaction with the 'specific' site in pRB.

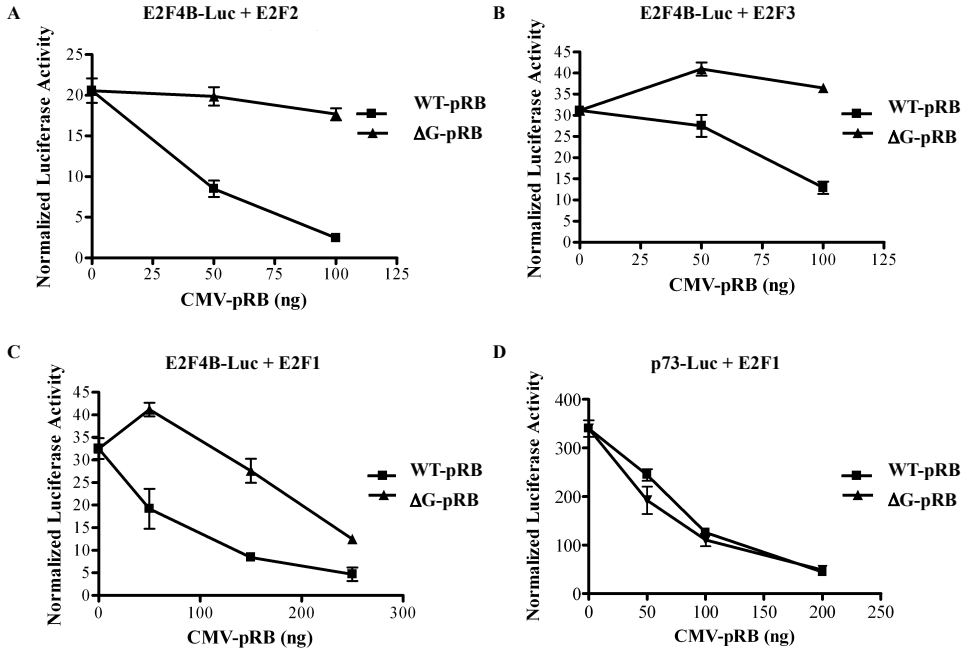
Given the requirement of the 'specific' site for the complex between ppRB and E2F1, the possibility that the P329 in E2F3 functioned to block the interaction with ppRB was investigated. In a similar manner to previous experiments, WT-E2F3 is unable to interact with ppRB and only the residual hypophosphorylated species is precipitated by HA-E2F3 (Fig. 2.6e). However, substitution of P329V in E2F3 results in an enhanced interaction with ppRB as it is capable of immunoprecipitating pRB phosphorylated at S807/S811 that is partially shifted in migration (Fig. 2.6e). This further confirms that the

interaction with ppRB requires the ability to bind to the 'specific' site of pRB and this is blocked by the presence of a proline in the marked box domain of E2F3.

Taken together this provides novel insight into the mechanism by which E2F1 is capable of forming a unique interaction with the C-terminus of pRB. The 'specific' site contains hydrophobic contact sites that interact with a cleft that is formed by both E2F1 and DP1. The specificity of this site for E2F1 is imparted by V276, since this amino acid is a proline in other E2Fs. The proline may create a conformation in the marked box in these E2Fs that does not interact with the 'specific' site. This proline in E2F3 also prevents the interaction with ppRB, thus supporting the importance of the 'specific' site in forming the E2F1-ppRB complex.

2.4.4 The 'specific' site maintains the ability to regulate the transcriptional activity of E2F1

The 'specific' site is resistant to disruption by phosphorylation and provides a means for pRB to selectively interact with E2F1 beyond the G_1 phase of the cell cycle. We sought to investigate the function of these complexes by testing the ability of the 'specific' site to regulate E2F dependent transcription. Saos-2 cells were transfected with HA-E2Fs (and DP1) and a plasmid encoding luciferase under the control of a canonical E2F response element (pE2F4B-Luc). As shown in figure 2.7a and 2.7b, HA-E2F2 and HA-E2F3 can both function as potent activators to stimulate transcription of luciferase from a reporter containing an E2F response element. Co-transfection of increasing amounts of WT-pRB results in a dose-dependent decrease in this activity (Fig. 2.7a,b).



(A) The ability of the pRB mutants to control E2F transcriptional activity was measured by luciferase reporter assays. Saos-2 cells were transfected with luciferase reporter plasmids, a CMV-b-gal plasmid and the indicated CMV-RB and –E2F expression plasmids. Extracts were prepared two days later and luciferase activity was normalized to b-gal activity. The E2F4B-Luciferase construct was co-transfected with E2F2 (A), E2F3 (B) or E2F1(C) to assess the ability of pRB to control transcription of individual E2Fs. (D) A reporter construct containing the p73 promoter termed p73-Luc was transfected with E2F1 and the pRB mutants to assess the ability of pRB to regulate a relevant target of E2F1. Each data point represents three independent transfections with error bars indicating one standard deviation from the mean

Figure 2-7 Transcriptional regulation of E2Fs by pRB through the 'general' and 'specific' E2F binding sites

This indicates that the WT-pRB is capable of repressing the transcriptional activity of E2F2 and E2F3. In contrast, the ΔG mutant is unable to repress the transcription of E2F2 or E2F3 even at the highest level of expression (Fig. 2.7a,b), which is consistent with the inability of ΔG to interact with E2F2 or E2F3 (Fig. 2.3d).

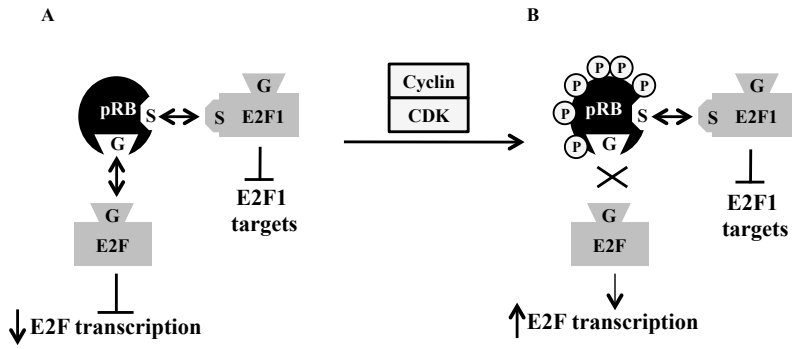
In a similar experiment, WT-pRB was shown to regulate the transcriptional activity of E2F1 (Fig. 2.7c). However, ΔG-pRB is also capable of regulating the activity of E2F1, albeit to a lesser extent than the WT-pRB as only the highest expression levels of ΔG-pRB affect transcription (Fig. 2.7c). This agrees with the ability of the 'specific' site to maintain the interaction with E2F1 in ΔG-pRB (Fig. 2.3d). To test the possibility that the 'specific' complex between pRB and E2F1 may regulate selective target genes, a luciferase construct containing the p73 promoter was utilized (p73-Luc). p73 is a well-studied target of E2F1 and activation of E2F1 by DNA damage has been shown to enhance the interaction of E2F1 with this promoter (*25, 31*). Strikingly, ΔG-pRB was found to regulate the activation of the p73 promoter to a similar extent as WT-pRB (Fig. 2.7d). This suggests that pRB-E2F1 complexes formed through the 'specific' site have the ability to regulate the expression of particular E2F1 target genes. This implies that ppRB-E2F1 complexes present in S-phase, or later in the cell cycle, are capable of negatively regulating E2F1-specific transcriptional targets.

2.5 Discussion

Contrary to current understanding of pRB-E2F regulation, this study suggests that ppRB can maintain an interaction with E2F1. Our data indicates that E2F1, but not E2F3, is capable of forming an interaction with ppRB, and that this interaction is dependent on

the ‘specific’ site of pRB. This suggests a potential mechanism by which phosphorylation can independently regulate the interaction between pRB and distinct E2F proteins (shown in Fig. 2.8). In its hypophosphorylated state, depicted in figure 2.8a, pRB is capable of interacting with E2Fs using the ‘general’ site or the ‘specific’ site. Phosphorylation of pRB by CDK complexes results in well described structural changes that disrupt E2F binding to the ‘general’ site (*9, 10, 14, 42, 45*). Our data, however, indicates that CDK phosphorylation does not disrupt the ‘specific’ site found in the C-terminus of pRB, and as a result, ppRB is capable of maintaining an interaction with E2F1. While previous studies have suggested the existence of E2F1 complexes with phosphorylated pRB, the mechanism by which phosphorylation of pRB could both disrupt interactions with some E2Fs and maintain interactions with E2F1 has been unknown. Our data provides a mechanism that explains the ability of pRB to be phosphorylated on most CDK directed sites while disrupting only a portion of pRB-E2F complexes.

This study describes the mechanistic basis for the unique ability of E2F1 to interact with ppRB. The ‘specific’ site of pRB, which is required for the interaction between ppRB and E2F1, provides the observed selectivity for E2F1 complexes. The selectivity of the ‘specific’ site is mediated in part by a valine at position 276 in E2F1. All other mammalian E2Fs, and E2Fs from multiple lower organisms, contain a proline at the analogous position to V276 in E2F1. This suggests that the ancestral E2F protein contained a proline at this position. During the divergence of E2F1 from the ancestral E2F proteins it is likely that the P276V substitution occurred and this was key to introducing a new interaction site between E2F1 and pRB. This sequence difference



(A) E2F1, E2F2, E2F3 and E2F4 utilize the 'general' or 'G' interaction to bind to hypophosphorylated pRB. This interaction inhibits E2Fs and decreases E2F dependent transcription. In addition to the 'general' interaction, E2F1 can interact with the 'specific' site (abbreviated as 'S') of pRB and this interaction is also capable of blocking activation of E2F1 targets. (B) Phosphorylation of pRB by Cyclin/CDK complexes results in a series of conformational changes that prevents the 'general' site from interacting with E2F transcription factors resulting in an increase in cell cycle gene expression. In contrast, the 'specific' site is not disrupted in ppRB and allows it to interact with E2F1 to regulate transcription.

Figure 2-8 Regulation of pRB-E2F interactions by phosphorylation

provides the means by which E2F1 can be regulated distinctly from other E2Fs and may have further promoted the divergence of E2F1 function from other E2Fs. E2F1 has a unique role in the induction of apoptosis that is not observed in other E2F transcription factors. This ability has raised the question as to how E2F1-induced apoptosis is attenuated in normal proliferating cells. Traditional models of pRB-E2F regulation suggest that all E2Fs are released from pRB following its phosphorylation at the G_1-S transition. The ability of cells to maintain viability as they proceed through the cell cycle suggests that additional mechanisms exist to inhibit the pro-apoptotic potential of E2F1. Our work has refined this model such that the roles of the two distinct E2F binding sites are included. As shown in figure 2.8 the 'general' site is disrupted by CDK phosphorylation but ppRB maintains the ability to interact with E2F1 through the 'specific' site. Thereby, phosphorylation of pRB results in the release of E2Fs from the 'general' site, which drive the expression of cell cycle genes, but maintains the interaction of E2F1 with the 'specific' site, which is capable of regulating E2F1 target genes.

While the 'specific' site is capable of regulating the transcriptional activity of E2F1, this complex has a relatively low affinity for the canonical E2F DNA response element (*15*). The ability of the 'specific' site to effectively control the activity of the p73-Luc promoter suggests that the 'specific' site may target pRB-E2F1 complexes to distinct regions of the genome. This is supported by work that identified targets for pRB and E2F1 in S-phase of the cell cycle (*30*). Cells synchronized in S-phase in that report contained largely ppRB that could be immunoprecipitated on DNA with phospho-specific antibodies. Taken with the results from our study, it suggests that the observed complexes

consisted of E2F1 bound to the 'specific' site of ppRB. Interestingly the targets of pRB in S-phase were not observed in other stages of the cell cycle suggesting that ppRB was capable of localizing to a distinct set of cellular genes (*30*). Thereby, phosphorylation of pRB results in complex formation between ppRB and E2F1 through the 'specific' site that may impart an altered DNA binding specificity and the regulation of distinct cellular targets.

Given that phosphorylation of pRB largely abrogates E2F binding and blocks the formation of pRB complexes at E2F-target genes few studies have investigated the ability of ppRB to maintain interaction with chromatin-remodeling factors. The ability of ppRB to maintain the interaction with E2F1 described in this study raises important questions regarding the ability of ppRB-E2F1 complexes to recruit chromatin remodeling factors to E2F1-target genes. Recent work has shown phosphorylated pRB at apoptotic promoters including p73 in response to DNA damage along with E2F1 and the histone acetyl transferase P/CAF (*31*). Interestingly the histone deacetylase HDAC1, which is commonly found associated with pRB on cell cycle promoters, is absent from the p73 promoter (*31*). This suggests that the phosphorylation state of pRB may allow for the recruitment of distinct chromatin remodeling complexes to E2F target genes. Furthermore, given the distinct interaction surfaces used in the 'general' and 'specific' complexes it is possible that these two complexes are capable of associating with different chromatin remodeling factors to give rise to the observed selectivity. Lastly, the 'specific' complex containing ppRB and E2F1 may serve as a platform on which to assemble activating or repressive complexes depending on growth status or other cell signals.

The majority of human cancers express functional but inactivated pRB that is maintained in a hyperphosphorylated state. The ability of ppRB to interact with E2F1 suggests that tumorigenesis may select for cells that maintain pRB in a hyperphosphorylated state as a means to attenuate E2F1-induced apoptosis while simultaneously deregulating proliferation. In some cases depletion of pRB in cells that express predominantly ppRB results in cell death (*46*), suggesting that the therapeutic disruption of the 'specific' site may provide a means to induce apoptosis in cancer cells expressing predominately ppRB. In contrast to the retention of wild type pRB, the majority of human tumors directly inactivate p53 to block the induction of apoptosis (*3*). The ability of p53-deficient cancer cells to undergo apoptosis is largely mediated by the p53 homologue p73, which is strongly activated by E2F1 (*47*). This further highlights the therapeutic potential of the 'specific' site as it could be utilized as a robust means to sensitize cancer cells to p73-dependent apoptosis. Taken together this work advances our understanding of the regulation of pRB-E2F interactions by CDK phosphorylation and suggests a selective advantage for retention of wild type pRB during tumorigenesis.

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3 The retinoblastoma tumor Suppressor Protein Engages Multiple Overlapping Pathways to Regulate Cell Cycle Entry.

3.1 Abstract

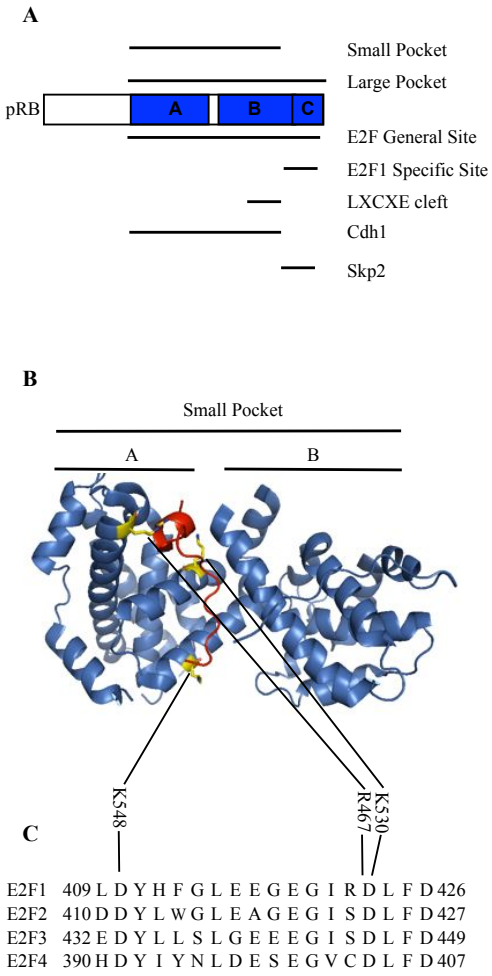
The retinoblastoma tumor suppressor protein (pRB) regulates the activity of E2F transcription factors to control entry into S-phase. Recent work has identified pRB regulation of p27 as an E2F independent mechanism of growth control. A second E2F binding site that is unique to E2F1, has also been identified in pRB that may also regulate proliferation. However, the contribution of these distinct interactions to the overall activity of pRB is not well understood because they have yet to be compared in a single study. We report the development of two pRB mutants that selectively disrupt individual protein interactions with pRB. One mutant substitutes R467E and K548E to disrupt E2F interactions with the small pocket region of pRB. The other substitutes Y756W to disrupt LXCXE type interactions with Cdh1 and HDACs. These mutants were utilized in combination with other mutations to systematically disrupt the known binding sites in pRB. Importantly the disruption of all E2F binding along with LXCXE interactions was sufficient to completely abrogate the ability of pRB to regulate cell cycle. When examining individual interactions, only loss of the 'general' E2F binding site results in even a partial decrease in the ability of pRB to arrest Saos-2 cells. Surprisingly, this

suggests redundant roles for the regulation of E2Fs and p27 in proliferative control. Taken together, pRB can engage multiple mechanisms through distinct binding interfaces to induce a cell cycle arrest.

3.2 Introduction

The retinoblastoma tumor suppressor gene (*RB1*) was the first identified tumor suppressor, and inactivation of the RB pathway is a common feature of essentially all types of cancer (*1, 2*). The RB protein product (pRB) is a key regulator of entry into the cell cycle and can control proliferation through the regulation of E2F target gene expression (*3*). pRB directly interacts with E2F transcription factors and blocks their ability to activate the genes required to progress into S-phase of the cell cycle. The interaction is mediated by a well-conserved region of the pocket domain of pRB that binds to the C-terminal transactivation domain of E2Fs (*4, 5*). The complex between pRB and E2Fs remains capable of binding to DNA and allows pRB to recruit chromatin remodeling factors through LXCXE binding cleft dependent interactions to participate in transcriptional repression of E2F target genes (*6-8*). The distinct binding sites are outlined in figure 3.1a.

In addition to controlling E2Fs, pRB is capable of regulating the G_1-S transition through the regulation of p27 levels. This regulation is mediated by the ability of pRB to bind to both Cdh1 and Skp2 to target Skp2 for degradation (*9*). Degradation of Skp2 prevents it from targeting p27 for destruction that in turn leads to increased p27 levels to block cyclin dependent kinase activity. Furthermore, pRB's ability to interact with Skp2 competitively inhibits Skp2-p27 interactions (*10*). The interaction with Cdh1 is mediated



(A) Diagram of the pocket domain structure of the pRB protein is shown at the top. The location of the A, B, and C regions is shown. The minimal fragments of pRB that mediate the indicated binding interactions are highlighted. (B) The structure of pRB (blue) in complex with E2F1 (red) (PDB:1O9K). Surface exposed residues of pRB that contact conserved residues in the E2F1 derived peptide are highlighted in yellow. (C) Conservation of the co-crystallized E2F1 peptide sequence amongst other E2F transcription factors. Conserved interactions between K548, K530, and R467 and acidic amino acids in E2Fs are indicated by lines.

Figure 3-1 Rationale and design for a novel ΔG-pRB mutant

in part by the LXCXE binding cleft contained within the B region of pRB. Mutants of pRB that disrupt other functions of pRB including the partially penetrant R661W allele remain capable of regulating proliferation through the Cdh1-Skp2-p27 axis (*9, 10*), suggesting that this regulation has a crucial role in cell cycle regulation.

In addition to regulating proliferation, pRB has the ability to negatively control E2F1 induced apoptosis. E2F1 is unique amongst the E2F transcription factors as it has the ability to induce both proliferation and apoptosis. This necessitates a mechanism by which E2F1-induced apoptosis can be restrained in normal proliferating cells. Recently it has been shown that pRB contains two distinct E2F binding sites that can control these contrasting functions (*11-13*). The first binding site termed the 'general' site is capable of interacting with E2F1-4 and is described above to have a critical role in controlling E2F induced proliferation. The second binding site forms a unique interaction with E2F1 near the C-terminus of pRB and it is known as the 'specific' site. While the 'specific' E2F1 interaction can potently block E2F1 induced apoptosis, disruption of this interaction in combination with other mutations in pRB suggests it can affect proliferative control under certain circumstances (*13*).

Much of our understanding of pRB's mechanism of action in G_1 regulation has come from studying its re-expression in the *RB1* deficient osteosarcoma cell line, Saos-2 (*9, 10, 14-22*). From studies using re-expression of pRB we have come to appreciate that it represses E2F transcription coincident with a G_1 arrest (*23*). Interestingly, experiments that demonstrate the cell cycle regulatory properties of Cdh1 and Skp2 in proliferation use Saos-2 based cell lines with tetracycline inducible pRB expression. This raises the question of whether pRB uses different arrest mechanisms (E2F vs. Cdh1-Skp2) based on

an unknown difference in cellular context of these experiments, or if both mechanisms co-exist in a single cell cycle arrest event. Furthermore, pRB has been shown to form mutually exclusive complexes with E2F1 and Cdh1 (*24*) suggesting that there may be some level of competition between these mechanisms of cell cycle arrest. Taken together it is unclear how these pathways co-exist in proliferative control.

In this report we sought to investigate the relative contributions of E2F and Cdh1-Skp2 regulation in cell cycle control. We describe the development of a number of pRB mutants with discrete defects in interacting with E2Fs or chromatin regulators and Cdh1. This has allowed us to establish a map of the protein interactions necessary for pRB-induced arrest of Saos-2 cells. Surprisingly, no single contact site on pRB is absolutely essential for proliferative control. Instead, our work suggests that cell cycle control by pRB is carried out through a redundant mechanism that incorporates E2F regulation of transcription and Cdh1-Skp2 control of cyclin/cdk activity simultaneously.

3.3 Methods

3.3.1 Plasmid constructions

Site-directed mutagenesis of a pRB cDNA was carried out by PCR as previously described (*17, 25*). Mutants were introduced into the bacterial GST-RBLP (Large Pocket Domain) expression vector pscodon (Delphi Genetics). All subclones of PCR products were sequenced to ensure that they only contained the desired mutations. Mutants were cloned as an AccI/NheI fragment into the CMV-pRB expression construct. CMV-HA-E2F1, -E2F2, E2F3 and E2F4, CMV-HA-DP1, CMV-TAg, CMV-β-Gal, CMV-CD20,

pE2F4B-Luc p107(-280)-Luc and pBB14 and their sources have been described previously (*12*). The CMV-Myc-Cdh1 expression plasmid was a generous gift from Nick Dyson (MGH, Boston, MA, USA).

3.3.2 Cell Culture

Saos-2, C33A and HeLa cell lines were obtained from ATCC and cultured according to standard methods (*17*). Cell culture was carried out in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2mM L-glutamine, penicillin (50U/mL) and streptomycin (50μg/mL). The C33A cells were used to study protein interaction to generate extracts for GST-pulldown and co-transfection immunoprecipitation experiments as well as for the E2F1 induced apoptosis assay. C33A cells were transfected using calcium phosphate with the precipitates left on the cells for 16h before fresh growth medium was added. The Saos-2 cells were transfected with Fugene 6 (Roche) as recommended by the manufacturer. These cells were used for the luciferase reporter and the cell cycle arrest assays.

3.3.3 Immunoprecipitations and western blotting

GST-pull-down and co-immunoprecipitation assays were performed as previously described (*17*) except figure 3.5b where HeLa nuclear extracts were used as described in Isaac, et. al. (*26*) and figure 3.6a which used the methods published in Binné et. al. (*9*). To generate extracts for these experiments C33A cells were seeded at 6×10^6 cells in 15cm dishes and transfected with a total of 60μg of DNA using calcium phosphate. 48 hours after transfection the cells were washed twice with phosphate buffered saline (PBS)

and collected into 1mL of Gel Shift Extract (GSE) buffer (20mM Tris pH7.5, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, 5μg/mL leupeptin, 5μg/mL aprotinin, 0.1mM Na_3VO_4, 0.5mM NaF and 1mM DTT). Extracts were freeze-thawed at -80 C and cellular debris was removed by centrifugation at 14,000 rpm. For co-transfection immunoprecipitations C33A extract was diluted in IP Wash buffer (20mM Tris pH 7.5, 200mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25mM DTT and 0.1% NP-40). pRB complexes were immunoprecipitated with 12CA5 (α-HA) for HA-tagged E2Fs or PAB419 (α-TAg), bound to protein G-sepharose (GE healthcare). Immunoprecipitations were incubated with rocking for 1 hour at 4°C. The protein G-sepharose beads were washed twice with IP wash buffer then resuspended in 1X-SDS-PAGE sample buffer and boiled at 95 C for 5 min to elute the bound proteins. The eluted material was resolved by electrophoresis on sodium dodecyl sulfate-8% polyacrylamide gels (SDS-8%PAGE) gel. Proteins were transferred to a nitrocellulose membrane by standard techniques. HA-tagged E2Fs were detected by the 12CA5 monoclonal antibody, Myc-tagged Cdh1 by 9E10, TAg by PAb419, pRB by C36 or G3245 (BD Bioscience), Skp2 by sc-7164 (Santa Cruz), HDAC by sc-6298 (Santa Cruz), RbAp46 sc-8272 (Santa Cruz) and RBP1 by LY11.

3.3.4 GST pulldown binding experiments

GST-fusion proteins were expressed in BL21-DE3-Gold *E.coli* in 500mL cultures. Briefly, a 25mL culture of cells containing the GST-fusion protein cloned into either pscodon (Delphi Genetics) or pGEX 4T-2 were grown overnight at room temperature with ampicillin. The following day the 25mL culture was used to inoculate

500mL of LB media in a 2L flask. The cells were grown for 2 hours at room temperature after which 100μM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures for overnight growth and protein induction at 16°C. The following morning the cells were harvested and GST-fusion proteins were purified using glutathione sepharose according to standard protocols. Extracts for GST-pulldowns were prepared in a similar manner to the co-transfection immunoprecipitation experiments with transfected C33A cells. Purified GST-fusion proteins (2μg) were diluted in low salt GSE buffer (20mM Tris pH 7.5, 200mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 1mM DTT and 0.1% NP-40) and incubated with 100μL of C33A extract expressing E2Fs or other pRB interacting proteins. For Cdh1 interaction studies 100ng of GST-RBLP was used with 10μL of extract expressing myc-Cdh1. GST-pRB complexes were precipitated with glutathione sepharose and washed twice with low salt GSE buffer and eluted with 1X-SDS-PAGE sample buffer. Samples were subjected to western blot using the same antibodies outlined for the immunoprecipitation experiments. For interaction with CRFs in figure 3.5b HeLa nuclear extract was utilized. For GST-E7 pulldown experiments 2μg of recombinant GST-E7 was used to pulldown pRB from C33A cell extracts. The GST-E7 was a generous gift from Biljana Todorovic and Joe Mymryk (University of Western Ontario, London, Ontario)

3.3.5 Luciferase reporter assays

Transcriptional reporter assays were carried out as reported previously (*17*). Saos-2 cells were plated in 6 well plates at a density of 5 X 10^5 cells per well. Cells were transfected with 100ng of the E2F4B-luciferase reporter or 200ng of the p107-Luc

reporter plasmid along with 15ng of CMV-HA-E2F, 15ng CMV-HA-DP1 and 200ng of CMV-β-Gal. Increasing concentrations of CMV-pRB expression plasmid were transfected to block the activity of the transfected E2Fs. Total plasmid DNA was normalized with the addition of CMV-CD20. Cells were harvested 36 hours after transfection with 1X Reporter Lysis Buffer (Promega). The luciferase activity was determined and normalized to β-gal activity. The β-gal activity was determined using standard techniques to measure the hydrolysis of 2-Nitrophenyl β–D-galactopyranoside (ONPG) at 405nm. The average of three independent transfections is shown and the error bars indicate one standard deviation from the mean.

3.3.6 Flow cytometry assays

3.3.6.1 Cell Cycle

Saos-2 cells were transfected and harvested as previously described (*17*). Briefly 1 X 10^6 cells were plated in 6cm dishes and transfected with 0.75μg of CMV-pRB 1μg of CMV-CD20 and 3.25μg of CMV-β-gal or 1μg of CMV-CD20 and 4μg of CMV-β-gal using Fugene 6 (Roche). Cells were re-plated in 10cm dishes 24 hours after transfection and harvested 72 hours after transfection. Cells were stained with propidium iodide (PI) and fluorescein conjugated anti-CD20 antibody prior to flow cytometric analysis. The percentage of CD20 positive cells with 2N DNA content was quantified. The graph displays the average of three independent transfections and error bars represent one standard deviation from the mean.

3.3.6.2 Apoptosis

E2F1-dependent apoptosis was measured in transfected C33A cells as previously reported (*12*). One million cells were plated in 6cm dishes and transfected using calcium phosphate. 0.25μg of CMV-HA-E2F1 and CMV-HA-DP1 or 0.5μg CMV-β-gal was transfected along with 1μg of pBB14 (membrane bound GFP expression vector) and 8.5μg CMV-HA-pRB or CMV-HA-β-gal. Adherent and floating cells were harvested and stained with PI prior to analysis by flow cytometry. The population of GFP positive cells with less than 2N DNA content was quantified. All graphs display the average of three independent transfections and error bars represent one standard deviation from the mean.

3.3.7 Determination of protein stability

C33A cells were transfected with CMV-pRB mutant constructs using calcium phosphate in 15cm dishes at a density of 6 million cells per plate. The following morning the plates were washed twice with PBS and split onto 6, 6cm dishes. 24 hours later, 100μg/mL cycloheximide was added to each plate and plates were harvested at 0, 3, 6, 9, 12 and 15 hour time points. Extracts were prepared from the cells and 25μg of total protein was loaded in each lane of the gel. The relative levels of pRB were determined by western blotting using the pRB antibody G3-245 (BD Pharmingen).

3.4 Results

3.4.1 Disruption of the 'general' E2F binding site

The interaction of Cdh1 and Skp2 with pRB offers a relatively new and largely unexplored means by which pRB can control cell cycle advancement. Recent work by Binné et al. has revealed that the previously generated pRB mutant that is defective for E2F binding (called ΔG because it disrupts the 'general' E2F interaction), is also partially defective for Cdh1 binding (*9*). Because the precise interaction site of Skp2 on pRB is unknown, and the overlapping nature of all of these interactions with pRB, it is difficult to define how they each contribute to pRB's ability to control proliferation (Fig. 3.1a). To better understand the relative contribution of E2F and Cdh1 binding to overall growth control by pRB, we sought to develop a new mutant allele that selectively disrupts E2F binding to the pRB pocket domain.

We were aided by two crystal structures that define the interface between pRB and E2Fs at the 'general' E2F binding site (Fig. 3.1b)(*4, 5*). This binding site is formed in part by a cleft that is shaped by the A and B regions of the pocket domain of pRB (Fig. 3.1b). The co-crystallized E2F peptide contacts predominantly the A box of pRB through the ends of the bound peptide (*4, 5*). The amino acids in the E2F peptide that mediate this interaction with pRB are conserved among E2Fs that are known to bind to pRB (Fig. 3.1c). This suggests that these are key sites of contact between pRB and E2Fs. In an effort to minimize the number of mutations required to disrupt this interaction, and take advantage of the electrostatic nature of these conserved contacts, we introduced acidic

amino acids in place of well conserved basic residues in pRB. Specifically the following substitutions were generated; K530D, K548E and R467E.

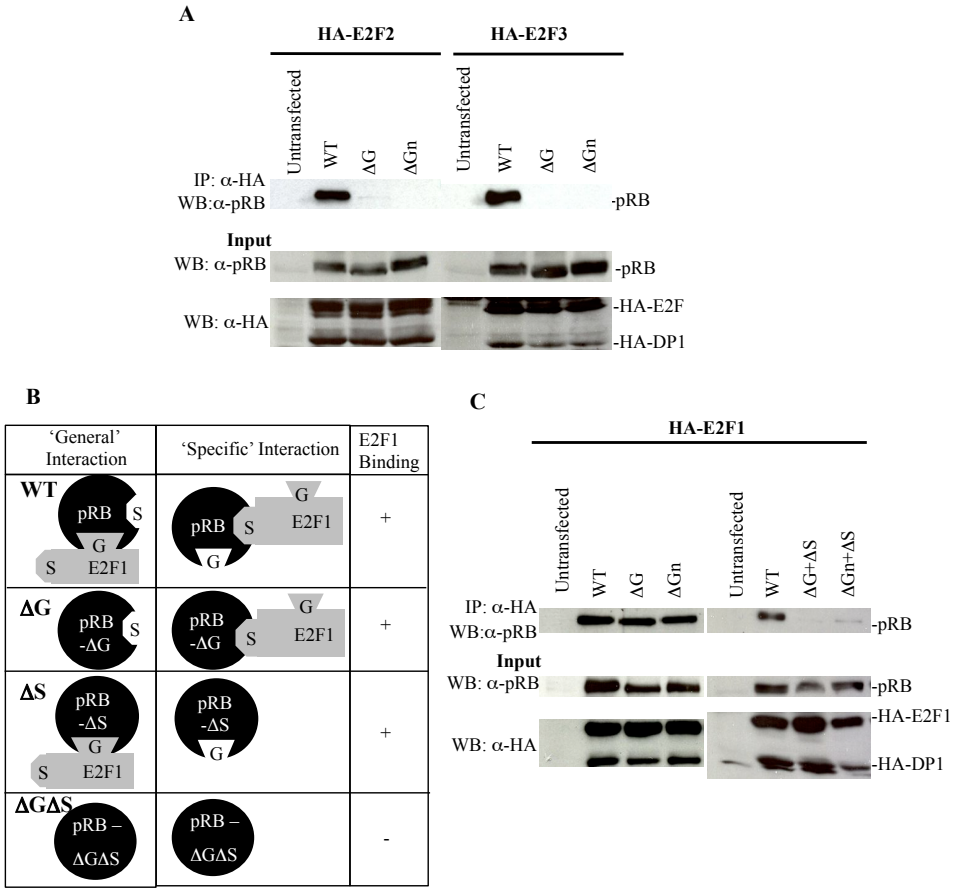
Mutation of R467E, K548E, or K530D individually were insufficient to abrogate binding of E2Fs at the 'general site' as determined by GST-pull down experiments summarized in Table 3.1. The R467E and K548E combination was sufficient to disrupt binding of E2F2 and E2F3 in immunoprecipitation-western blotting or GST-pulldown experiments (Fig. 3.2a). We define this set of mutations as ΔGn throughout the remainder of this study and use it to disrupt the 'general' E2F binding site. As outlined in figure 3.2b, E2F1 can bind to pRB through either of two distinct interaction sites. Disruption of the 'general' E2F binding site is insufficient to disrupt E2F1 binding to pRB (*12*), as E2F1 simply uses the 'specific' binding site instead (Fig. 3.2b) (*13*). In a similar manner the ΔGn mutant retained the ability to interact with E2F1 (Fig. 3.2c and Table 3.1). However, combining the ΔGn mutant with the previously reported mutant that disrupts the 'specific' site (ΔS) drastically reduces E2F1 binding (Fig. 3.2c). This indicates that the ΔGn mutation allows for the disruption of E2F1-4 binding to the 'general' E2F binding site on pRB, while leaving other distinct binding sites intact.

Taken together, these experiments reveal that the ΔGn mutant is ideal for studying E2F regulation, as this mutant has discrete defects in E2F interaction.

3.4.2 The ΔGn mutant is defective in controlling E2F dependent transcription.

In addition to disrupting the interaction of E2Fs with pRB the ΔGn mutant is defective in regulating the transcriptional activity of E2F transcription factors. A *p107*-Luciferase reporter construct was used because pRB regulates its transcription in an E2F dependent manner (*27*). In figure 3.3a WT-pRB is able to repress the *p107*-Luciferase construct in a dose dependent manner by blocking the ability of endogenous E2F transcription factors to activate the *p107*-luciferase reporter. Conversely, the ΔG and the ΔGn mutant did not repress *p107*-Luc construct activity, suggesting that the ability to control E2F dependent transcription is abrogated in the ΔGn mutant.

In order to dissect the ability of the pRB mutants to regulate the activity of specific activator E2Fs, the individual E2Fs were expressed and the ability of the pRB mutants to block their transcriptional activation of the E2F4B-Luciferase reporter was measured. The E2F4B-luciferase construct has been shown previously to measure E2F-activated transcription from 4 tandem E2F binding sites (*17, 28*). Figures 3.3b and c show that WT-pRB is able to repress E2F2 and E2F3 activity, but the ΔGn mutant is again unable to repress the transcription of either E2F2 or E2F3. Taken together, these data indicate that the ΔGn mutant and the ΔG mutant function similarly in that they do not repress endogenous E2F activity and that of ectopically expressed E2F2 or E2F3. We also investigated the transcriptional regulation of E2F1 by the ΔGn mutant (Fig. 3.3d). In this case, increasing levels of the pRB ΔGn mutant resulted in distinct repression of E2F1



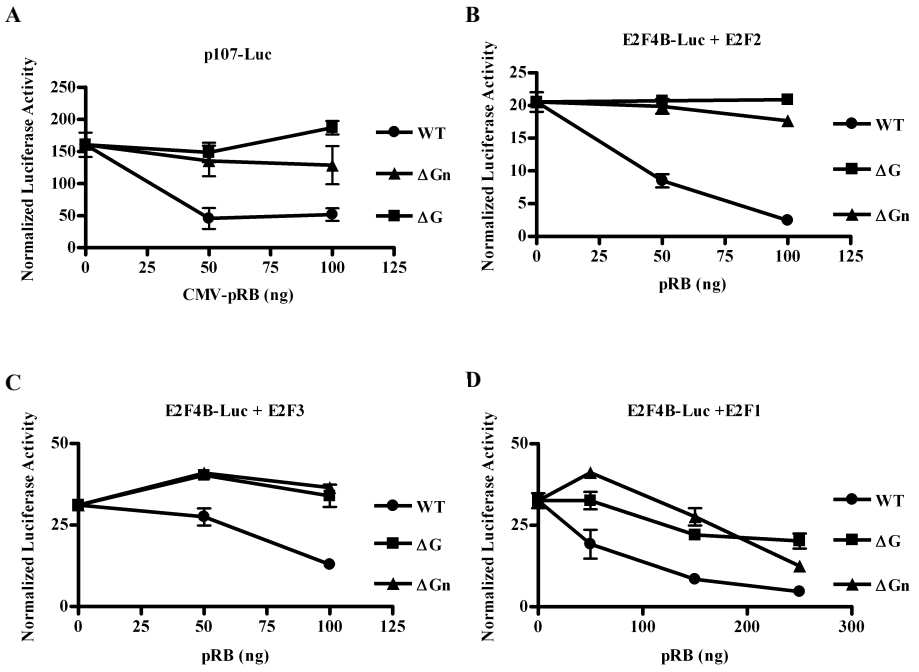
(A,C) C33A cells were co-transfected with expression plasmids for pRB and HA-tagged forms of E2F1, E2F2 or E2F3 with DP1. The lower western blots represent the input expression levels of the proteins in these extracts. The amount of pRB that co-precipitates with HA-E2F is shown in the upper panel. (B) Diagram outlining the two mechanisms by which E2F1 can interact with pRB through the 'general' or the 'specific' sites. The pRB DG mutant blocks the interaction at the 'general' while allowing E2F1 to bind to the 'specific' site. Combination of the DG and DS mutants disrupts all E2F1 binding to pRB. To study pRB binding to E2F1 through the 'general' site, the ΔS mutant was used to disrupt binding at only the 'specific' site. (C) This approach was used to characterize the effects of the R467E, K548E mutations in pRB on E2F1 binding.

Figure 3-2 The ΔGn mutant disrupts the 'general' interaction in the pRB pocket domain

Table 3-1 Summary of binding characteristics of new ΔG mutants

Mutant	E2F1	E2F2	E2F3	E2F4	TAg
ΔG	++	-	-	-	++
ΔG +ΔS	-	-	-	-	ND
R467E	++	++	++	-	++
K530D	++	++	++	-	++
K548E	++	++	++	++	++
ΔGn (R467E/K548E)	++	-	-	-	++
K530D/K548E	+	-	-	-	++
ΔGn+ΔS	-	-	-	ND	ND
K530D/K548E +ΔS	-	-	-	ND	ND

Summarized data obtained from both GST-pulldowns and co-transfection immunoprecipitation experiments with the pRB mutants. All binding is relative to WT-pRB. ++ Indicates binding equal to WT, + indicates reduced binding compared to WT, - indicates little to no detectable binding. ND: Not Determined.



(A) The ability of the pRB mutants to control E2F transcriptional activity was measured by luciferase reporter assays. Saos-2 cells were transfected with luciferase reporter plasmids, a CMV-b-gal plasmid and the indicated CMV-RB plasmids. Extracts were prepared two days later and luciferase activity was normalized to b-gal activity. (A) The ability of the pRB mutants to utilize endogenous E2Fs and repress a p107-Luc reporter was determined (B-D) The E2F4B-Luciferase construct was co-transfected with E2F1, E2F2, or E2F3 and CMV-pRB constructs to assess the ability of pRB to control transcription of individual E2Fs. Each data point represents three independent transfections with error bars indicating one standard deviation from the mean.

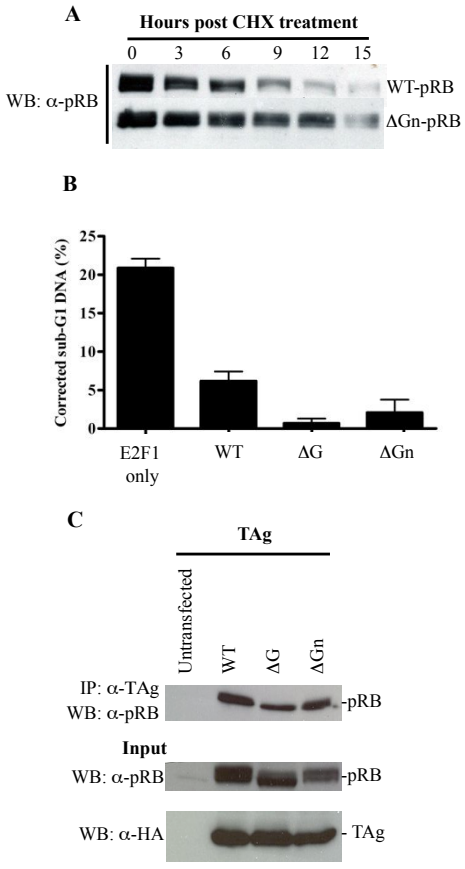
Figure 3-3 The ΔGn is defective in controlling E2F dependent transcription

activity in a dose-dependent manner. This effect is consistent with regulation occurring through the 'specific' E2F1 interaction site that remains in the ΔGn mutant. This effect was also observed with the ΔG mutant and its ability to regulate E2F1 separately from other E2Fs has been previously published (*16*). These experiments show that ΔGn pRB exhibits a discrete defect in regulating E2F transcription factors demonstrated by transcription reporter assays, as well as protein-protein interaction assays.

3.4.3 The pRB ΔGn mutant is stably expressed and other binding sites are maintained

We measured the stability of the ΔGn mutant because other pRB mutations have been found to disrupt overall protein stability. CMV expression vectors encoding WT-pRB or ΔGn-pRB were transfected into C33A and relative stability was monitored by blocking protein synthesis with cycloheximide. Levels of pRB were assessed by western blotting over a 15 hour time course (Fig. 3.4a) and the ΔGn mutant was found to be as stable as WT-pRB with an approximate half-life of 12 hours.

Previous studies have shown that the 'specific' site is required for pRB to block apoptosis induced by E2F1 (*13, 16*). Furthermore, disruption of the 'general' site enhances the ability of pRB to block apoptosis suggesting that when E2F1 is forced to bind to the 'specific' site there is an active role for this complex in repressing apoptosis. To further validate the activity of the 'specific' site we investigated its ability to regulate E2F1 induced apoptosis. As is shown in figure 3.4b the ΔGn mutant can repress E2F1-induced apoptosis equivalently to ΔG.



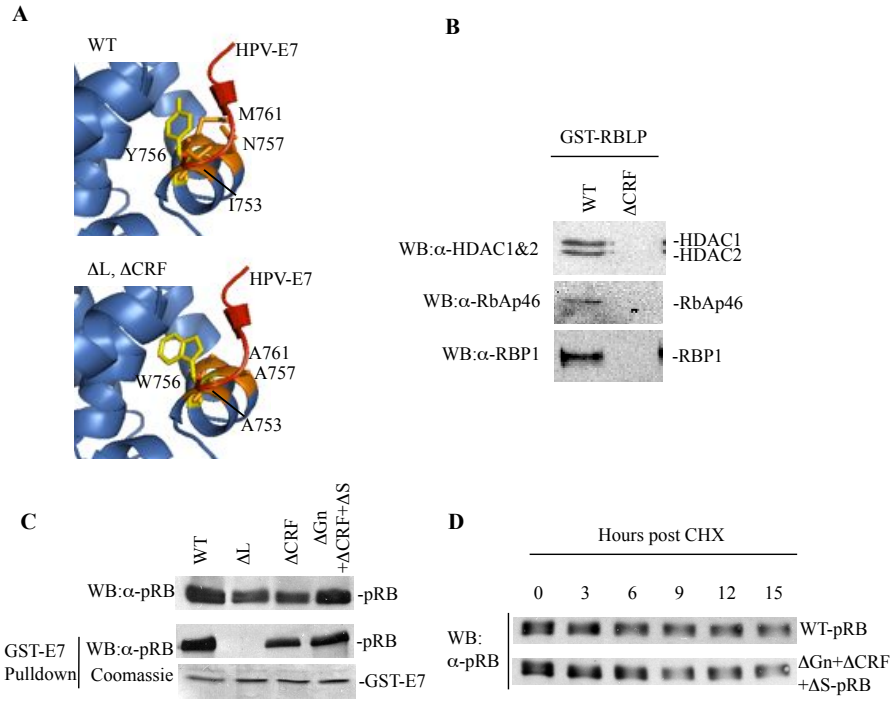
(A) CMV-pRB expression constructs were transfected into pRB deficient C33A cells and the stability of the transfected proteins was determined by inhibiting protein synthesis with cycloheximide. The level of transfected pRB was determined every three hours over a 15 hour time course by western blotting. (B) The ability of the E2F1 binding site in the ΔGn mutant to block E2F1 induced apoptosis was tested. C33A cells were transfected with pRB and E2F1/DP1 expression plasmids and a GFP reporter. Two days later the percentage of GFP positive cells with less than 2N DNA was measured using propidium iodide staining and flow cytometry. Each measurement is an average of three experiments and error bars indicate one standard deviation. (C) Plasmids expressing pRB-mutants and TAg were transfected into C33A cells and pRB was co-immunoprecipitated with TAg. Input levels of expressed proteins are shown in the lower panels and relative levels of bound protein are shown in the upper panel

Figure 3-4 Stability and apoptotic regulation by the ΔGn mutant

Lastly, to ensure that the LXCXE cleft is maintained in the ΔGn mutant, we also investigated the ability of TAg to bind to this pRB mutant in an immunoprecipitation and western blotting experiment (Fig. 3.4c). TAg is capable of forming a complex with the ΔGn mutant in an analogous manner to WT pRB and the ΔG mutant. Together, these experiments suggest that both the LXCXE binding cleft and the E2F1 'specific' site are maintained in the ΔGn mutant. Furthermore, the similar stability of the ΔGn mutant to wild type indicates it can selectively disrupt E2F binding without altering other aspects of pRB's function.

3.4.4 Selective disruption of LXCXE binding cleft dependent interactions with chromatin remodeling factors.

In order to create a pRB mutant that is functionless in cell cycle control, it is necessary to demonstrate that it is stable and capable of at least one type of protein interaction to rule-out a non-specific loss of function. To this end we generated a mutant that substitutes Y756 for tryptophan, termed ΔCRF. Y756 is found in the LXCXE binding cleft in the B region of the small pocket of pRB. It is found on the same helix as the M761, N757 and I753 residues that were substituted in the previously reported pRB ΔL allele that is defective for binding to chromatin regulators, such as HDACs, and viral proteins that use an LXCXE motif (*17, 25*). Figure 3.5a depicts a peptide containing the LXCXE sequence derived from the viral oncoprotein E7 bound to pRB (*29*). The ΔL substitutions shown in orange directly contact the E7 peptide while the Y756 residue fills part of the cleft that defines this binding site and does not directly contact the E7 peptide. The substitution of Y756W in the ΔCRF mutant is sufficient to disrupt the interaction



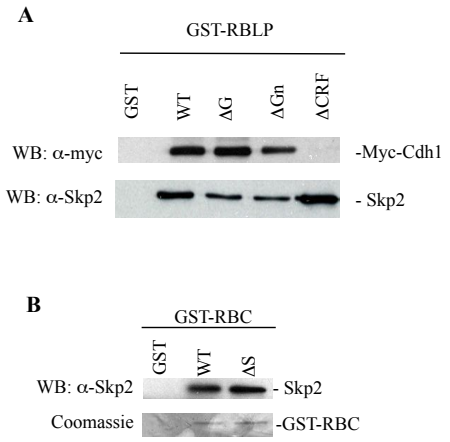
(A) The structure of pRB (blue) co-crystallized with a HPV-E7 peptide (red) bound to the LXCXE binding cleft (PDB: 1GUX). Substitutions in the ΔL mutant are shown in orange and the ΔCRF substitution is depicted in yellow. (B) Interaction of ΔCRF with chromatin remodeling factors. WT and ΔCRF GST-RBLP proteins were used to precipitate pRB interacting proteins from HeLa cells. Interacting HDAC1 and 2, RbAp46 and RBP1 were detected by western blotting. (C) Recombinant GST-E7 was incubated with extract containing pRB mutants and precipitated using glutathione sepharose. The relative amount of bound pRB was determined by western blotting. (D) Stability of WT-pRB and ΔGn+ΔCRF+ΔS-pRB expressed in C33A cells over a 15 hour time course in cells treated with cycloheximide.

Figure 3-5 The ΔCRF mutant selectively disrupts the interaction with chromatin remodeling factors

with chromatin remodeling factors including HDAC1, HDAC2, RbAp46 and RBP1 in a GST-pulldown assay (Fig. 3.5b). However, the interaction with viral oncoproteins is maintained in the ΔCRF mutant (Fig. 3.5c). The interaction with GST-E7 ensures that when combined with other mutations that disrupt protein-protein interactions with pRB, a single remaining interaction can be used to characterize the overall integrity of the pocket domain of pRB. As shown in figure 3.5c the ΔGn+ΔCRF+ΔS mutant retains the ability to interact with GST-E7 suggesting that the pocket domain is intact in this mutant. Furthermore, the stability of the ΔGn+ΔCRF+ΔS is not affected given that it has a similar half-life to WT-pRB when transfected into C33A cells (Fig. 3.5d). Taken together, the ΔCRF mutant selectively disrupts the interaction with chromatin remodeling factors while maintaining overall stability and structural integrity. These properties are critical to the interpretation of cell cycle arrest experiments using these mutants.

3.4.5 Selective abrogation of Cdh1 and pRB interactions using the ΔCRF mutant.

The retinoblastoma protein acts as a scaffold to bring together APC-Cdh1 and Skp2 leading to Skp2 ubiquitylation and degradation. Loss of interaction through the LXCXE binding cleft on pRB prevents APC-Cdh1 dependent degradation of Skp2 and is reported to cripple pRB dependent cell cycle arrest (*9*). In addition to disrupting the interaction with chromatin remodeling factors, the ΔCRF mutant greatly reduces the interaction with Cdh1. A GST-pulldown assay is shown in figure 3.6a examining the interaction properties of our pRB mutants with Cdh1 that demonstrates this property and also confirms that ΔGn is not deficient for this interaction. Since Cdh1 uses pRB to



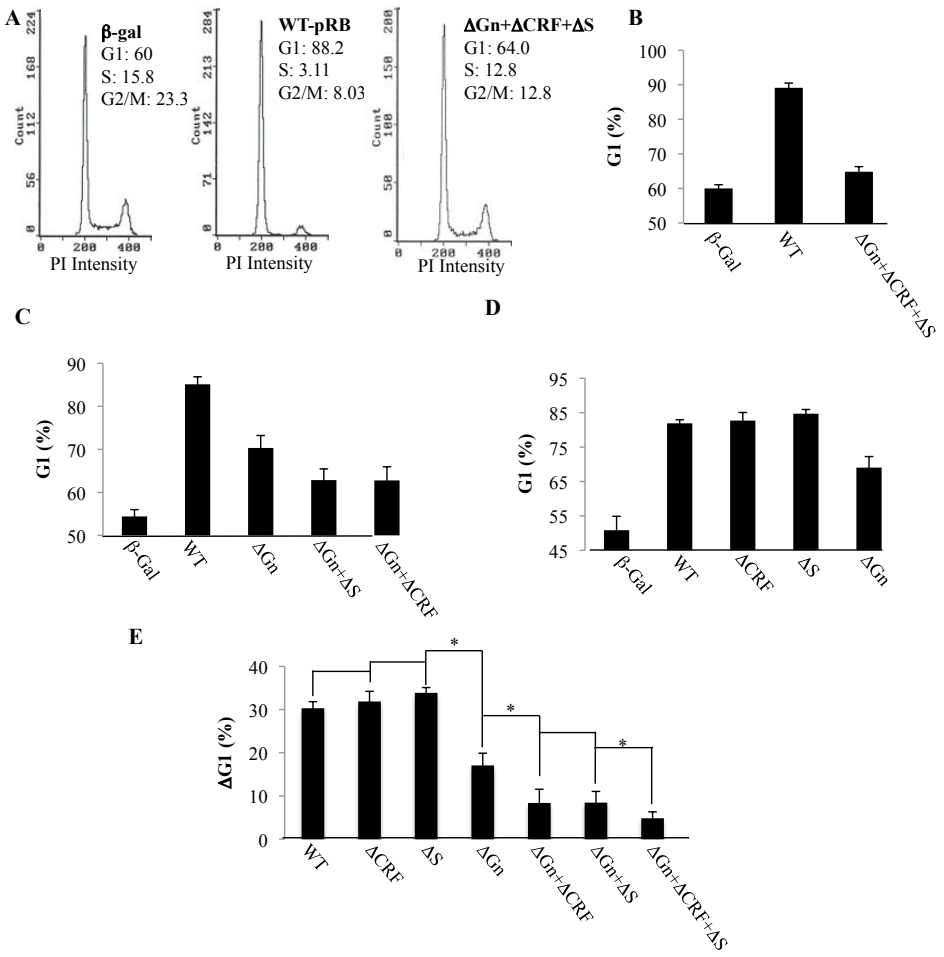
A) GST-RBLP mutants were incubated with C33A extracts containing myc-Cdh1. Co-precipitated proteins were immunoblotted for myc and Skp2 to assess the ability of the various RBLP mutants to interact with Cdh1 and Skp2. (F) GST-RBC ΔS mutant was incubated with C33A extracts. Co-precipitated proteins were immunoblotted for Skp2.

Figure 3-6 The ΔCRF mutant selectively disrupts the interaction with Cdh1

capture Skp2 it is also important to confirm that Skp2 binds to pRB normally. Since the precise binding site for Skp2 in the C-terminus of pRB is unknown and the ΔS substitutions that we are using are found within this part of pRB, we tested the ability of C-terminal fragments of pRB to interact with Skp2. Figure 3.6b shows that a C-terminal fragment of pRB (RBC) that contains the ΔS mutation interacts with Skp2 to a similar extent as the WT protein. In this way, the pRB ΔCRF mutant creates a discrete loss of Cdh1 binding, but pRB's ability to recruit Skp2 is retained in this C-terminal mutant. Therefore this mutation allows us to disrupt this aspect of growth control by pRB at the level of Cdh1 recruitment. This collection of mutants permits examination of the relative contributions of E2Fs through their two types of interaction with pRB as well Cdh1 targeting of Skp2 to cell cycle control.

3.4.6 Separating the relative contributions of E2F and Cdh1 interactions in cell cycle control by pRB.

The ΔGn mutant allows separation of E2F binding to the 'general site' from other functions of pRB including interaction with Cdh1. To investigate the contribution of these various binding sites to overall cell cycle control by pRB, we generated mutants that contained combinations of our ΔGn, ΔS and ΔCRF substitutions. The amino acid substitutions and properties of the mutant combinations are summarized in Table 3.2. The relative activity of these mutants to arrest proliferation was characterized using the well-studied Saos-2 cell cycle arrest assay. CMV-pRB was expressed at the minimum level needed to achieve maximal growth suppression; the CMV-pRB mutants were then expressed using the same conditions to assess their ability to control proliferation.



(A-C) Saos-2 cells were transfected with CMV-CD20 and CMV-pRB constructs and replated at low density to give the cells the ability to proliferate. Two days later cells were stained with an anti-CD20 flourescein conjugated antibody and propidium iodide. The percentage of cells with G1 DNA content, that were CD20 positive, was determined by flow cytometry. (A) Cell cycle distribution of CD20 positive cells transfected with b-gal, WT-pRB or ΔGn+ΔCRF+ΔS-pRB. (B-D) Graphical representation of the mean percentage of cells with G1 DNA content from at least three independent transfections. (E) Data from all experiments was compiled and compared directly by scaling pRB's relative cell cycle arrest ability to the change in the percentage of G1 cells. Error bars represent one standard deviation from the mean. * denotes a statistically significant difference between the indicated measurements using a t-test (P<0.05).

Figure 3-7 Multiple protein interactions are necessary for a G_1 arrest

Table 3-2 Disruption of distinct binding sites by pRB mutants

Mutant	Substitutions	E2F-'General Site'	E2F1-'Specific Site'	Viral protein Binding	Chromatin Remodeling Factors	Cdh1	Skp2
ΔGn	R467E K548E	-	++	++	ND	++	++
ΔG	S463A E464A R544A K548A K652A R656A L660A T664A R668A K873A K874A	-	++	++	ND	++	++
ΔCRF	Y756W	++	++	++	-	-	++
ΔS	M851A V852A	++	-	++	ND	ND	++
ΔGn+ΔCRF	R467E K548E Y756W	-	++	++	ND	-	++
ΔGn+ΔS	R467E K548E M851A V852A	-	-	++	ND	ND	ND
ΔGn+ΔCRF+ΔS	R467E K548E Y756W M851A V852A	-	-	++	ND	ND	ND

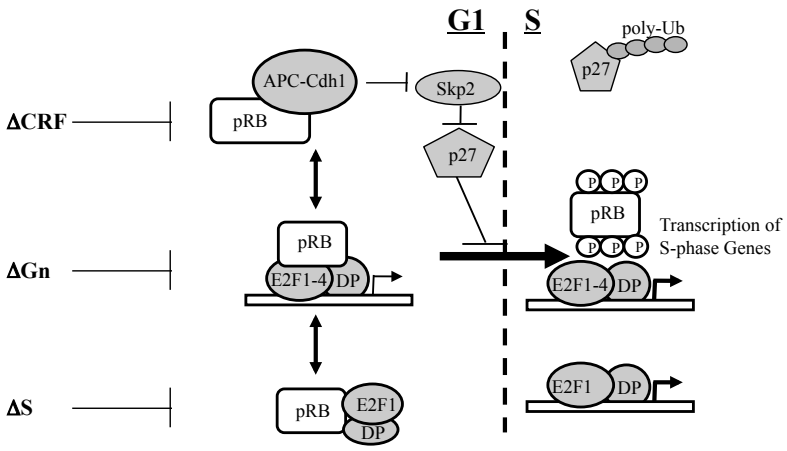
++ denotes binding site is intact, + indicates the binding site is partially disrupted and – indicates that the binding is undetectable. ND: Not Determined.

Combined disruption of both types of E2F interaction and the LXCXE binding cleft in the ΔGn+ΔCRF+ΔS mutant completely disrupts the ability of pRB to regulate proliferation given that it is not statistically different from the β-gal negative control (Fig. 3.7a and b). This suggests that the E2F and LXCXE binding sites mediate the cell cycle control activity of pRB dectectable by this assay. Surprisingly, disruption of the 'general' E2F binding site in the ΔGn mutant is sufficient on its own to reduce pRB's ability to block proliferation (Fig. 3.7c and d). Combined disruption of the 'general' site along with disruption of the LXCXE binding cleft (ΔCRF) or disruption of the E2F1 'specific' site (ΔS) resulted in a further decrease in the ability of pRB to induce a cell cycle arrest (Fig. 3.7d). Interestingly, neither the ΔCRF nor the ΔS mutations alone compromise pRB's ability to control cell cycle advancement. Each of these experiments were assessed side-by-side to facilitate *t*-test analyses (Fig. 3.7e). All of the above mentioned differences are statistically significant ($P<0.05$).

These results reveal a surprising degree of flexibility by pRB in growth control whereby it can engage multiple growth suppressive pathways as needed. In particular, some of these pathways are only required when others are compromised.

3.5 Discussion

pRB acts as an adapter protein to interact with various cellular proteins through distinct binding surfaces to control cell proliferation. In this report we describe the generation and combination of mutants that allow us to discretely and quantitatively account for pRB's growth suppression activity. To quantify the activity of pRB we



pRB is capable of forming at least three distinct interchangeable complexes to regulate proliferation. The control of E2F transcription factors through the 'general site' is the dominant mode of cell cycle control mediated by pRB because its absence causes a partial loss of proliferative control. In the absence of the 'general' E2F site the interaction with Skp2 and Cdh1 or the interaction with E2F1 through the 'specific site' can act to maintain proliferative control by pRB.

Figure 3-8 Model of pRB proliferative control

expressed the pRB mutants into Saos-2 and measured the percentage of cells arrested in G_1. We find that disruption of both types of E2F interactions and p27 regulation is required to fully abrogate cell cycle control by pRB. This suggests that pRB utilizes a number of distinct mechanisms to control cell cycle arrest as depicted in figure 3.8. The regulation of E2Fs through the pRB 'general' site appears to have a dominant role in cell cycle control because the ΔGn mutant was the only single mutant that altered proliferative control by pRB. The 'general' E2F binding site of pRB functions by interacting with and blocking the transactivation region of E2F1-4. The dominant nature of E2F regulation fits with the essential role for activator E2Fs in proliferation, as fibroblasts lacking E2F1-3 are unable to enter into S-Phase (*30*) and E2Fs are required for proper development in mice (*31*). The mechanism by which E2F regulation has a dominant role may involve the mutually exclusive nature of E2F and Cdh1 complexes with pRB that was recently described (*24*).

While the 'general' E2F binding site appears to have the most prominent role in controlling proliferation, its loss in isolation still leaves pRB with greater than 50% activity in our assays. For this reason, the LXCXE binding site and the 'specific' E2F1 binding site also have important roles in cell cycle regulation even though they appear redundant with other growth arresting mechanisms. This is consistent with the fact that fibroblasts derived from mice carrying a mutation in the *Rb1* gene that disrupts the LXCXE binding cleft have normal cell cycle entry control (*26*). Chromatin remodeling factors, such as HDACs, interact with the LXCXE binding cleft and are recruited to promoters through pRB in an E2F dependent manner. Disruption of E2F binding in ΔGn pRB will leave the interaction with CRFs intact but prevent their recruitment to E2F

target genes. Since disruption of the LXCXE binding cleft by the ΔCRF mutant in conjunction with the ΔGn substitution acts to further reduce the activity of pRB, it is unlikely that the added effect of LXCXE disruption is mediated by CRFs. pRB can however, regulate the levels of p27 independently of E2F activity through the interaction with Cdh1 and Skp2. The interaction with Cdh1 is greatly reduced in the ΔCRF mutant of pRB, which in turn disrupts the ability of pRB to regulate the levels of Skp2, and in turn p27 levels (*9*). Since functions associated with the LXCXE binding cleft occur independently of E2F binding we suggest that LXCXE motif interactions are critical to control cell cycle in the absence of E2F binding in our assays.

However, disruption of these two distinct pathways is insufficient to completely abrogate the activity of pRB, as the ΔGn+ΔCRF is still capable of inducing a partial arrest of Saos-2 cells. The remaining activity has been attributed to the E2F1 'specific' site found in the C-terminus of pRB. This site forms a unique interaction with the marked box region of E2F1. The complex between pRB and E2F1 bound through the 'specific' site was found to have a low affinity for DNA (*12*) and relatively weak regulation of E2F1 dependent transcription (Fig. 3.3d). This suggests that the site may function by sequestering E2F1 from E2F target genes to block cell cycle advancement or it may use a mechanism that is currently unappreciated.

The ability of pRB to engage multiple independent mechanisms of cell cycle arrest has important implications for why it is a barrier to oncogenic transformation. Our model predicts that disruption of proliferative control requires inactivation of three distinct binding interfaces on pRB. For this reason it is noteworthy that pRB is most often

inactivated in cancer by large deletions or the introduction of nonsense mutations that inactivate the entire protein (*32*). These types of mutations are the only way to simultaneously disrupt all elements of cell cycle control by pRB.

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4 E2F regulation by the retinoblastoma tumor suppressor protein is dispensable for proliferative control and tumor suppression

4.1 Abstract

The retinoblastoma tumor suppressor protein (pRb) has a well-described role in the regulation of the G_1-S phase transition of the cell cycle. In the G_1 phase of the cell cycle, pRb interacts with E2F transcription factors to attenuate the activation of S-phase target genes. To investigate the role of E2F regulation by pRb in proliferative control we generated a gene-targeted mouse model that introduced R461E and K542E substitutions into the endogenous *Rb1* gene. This allele, termed ΔG, disrupts the interaction between the large pocket domain of pRb and E2Fs. Fibroblasts derived from the $Rb1^{\Delta G/\Delta G}$ embryos have deregulated E2F target gene expression, and pRb-ΔG is defective in forming complexes with E2F response elements in gel shift assays. E2F target gene expression is deregulated in $Rb1^{\Delta G/\Delta G}$ fibroblasts to a similar extent, as observed in $Rb1^{-/-}$ cells, further suggesting that pRb-ΔG is unable to effectively regulate E2Fs. Strikingly, proliferative control is largely maintained in the $Rb1^{\Delta G/\Delta G}$ fibroblasts. Viable $Rb1^{\Delta G/\Delta G}$ mice have been obtained that do not display other overt phenotypes and do not appear to develop tumors. While $Rb1^{-/-}$ animals do not survive past E15 due to placental defects, $Rb1^{\Delta G/\Delta G}$ mice are born at the expected Mendelian ratios. Cell cycle regulation by pRB mutants deficient for E2F regulation was found to be dependent upon the LXCXE binding cleft, which can regulate p27 stability through the interaction with the Cdh1 subunit of the APC ubiquitin

ligase complex. To explore the maintained proliferative control, *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ fibroblasts were generated and appear to have distinct defects in maintaining proliferation. Furthermore, mice containing the ΔG mutation along with p27 deficiency rapidly develop pituitary tumors with a similar incidence to *Rb1*$^{+/-}$ *Cdkn1b*$^{-/-}$ animals. This suggests that deregulated E2F activity is not sufficient to induce ectopic proliferation, as pRb can regulate other pathways that include p27 stabilization to maintain cell cycle control and block tumorigenesis. These findings extend the molecular understanding of the tumor suppressive properties of pRb and suggest that direct regulation of E2F transcriptional activity by pRb is one aspect of its for cell cycle control and tumor suppression function.

4.2 Introduction

The retinoblastoma tumor suppressor protein (pRb) has a central role in the regulation of the G_1 checkpoint and inactivation of this control is a hallmark of cancer (*1*). pRb is thought to regulate the entry into S-phase largely through its ability to repress E2F transcription factors (*2*). E2Fs are potent transcription factors, which activate a transcriptional program to drive S-phase progression. In the G_1 phase of the cell cycle, a direct interaction mediated by the large pocket domain of pRb with the transactivation domain of E2Fs blocks the expression of S-phase target genes and is thought to maintain the cell in the G_1 phase of the cell cycle (*3*). Activation of cyclin-dependent kinases (CDK) results in the phosphorylation of pRb, which releases bound E2F transcription factors. The free E2Fs activate a transcriptional program, which drives the progression

into S phase and through the remainder of the cell cycle. This model of pRb function suggests that E2F regulation is a central element of proliferative control. This predicts that mutations that disrupt the binding to E2Fs should be tumorigenic. However, no mutations of pRb in cancer that have been shown to lead to discrete interaction defects (*4*). The mutations found in human cancers are typically complete loss of function alterations that completely inactive the pRb protein (*5*). The paucity of E2F-binding deficient mutants in human cancer suggests that other mechanisms may contribute to the tumor suppressive properties of pRb. However, the basis by which this may occur remains unclear.

In addition to the large pocket E2F-binding site defined as the 'general site', a second E2F-binding site (the 'specific site') has been identified in the C-terminus of pRb that forms a unique interaction with E2F1 (*6*). This site has been shown to function in the absence of the 'general site' to regulate proliferation to a small extent, but is thought to primarily function in the regulation of E2F1-induced apoptosis (*7*). Furthermore, E2F1 bound to the 'specific site' has a low affinity for the canonical E2F response element, yet retains the ability to regulate p73 transcription (*6, 8*). The 'specific site' is resistant to disruption by CDK phosphorylation suggesting that this site is regulated independently of the 'general site' to control the unique ability of E2F1 to induce apoptosis (*8*). However, the function of the 'general site' and 'specific site' at endogenous levels in non-transformed cells has yet to be characterized and development of a reagent to study these sites in isolation is needed to understand their contribution to proliferation and apoptosis.

In addition to the regulation of E2Fs, pRb is also capable of increasing the stability of the CDK inhibitor p27 (*9*). Specifically, pRb interacts with components of the

anaphase promoting complex (APC) when bound with the Cdh1 targeting subunit (*9*). This complex functions to promote the degradation of Skp2, and maintain cells in the G_1 phase of the cell cycle. pRb binds to Cdh1 through a binding site in the pocket domain and with Skp2 through the C-terminal domain (*9, 10*). By interacting with both of these proteins, pRb is capable of promoting the degradation of Skp2. This degradation prevents SCF^{Skp2} complexes from targeting p27 for degradation and results in the stabilization of p27(*9*). Cdh1 and E2F have been shown to form mutually exclusive complexes with pRb suggesting that there may be a complex interplay between growth suppressive pRb complexes in G_1 (*11*). While there is clear evidence that suggests pRb can regulate cell cycle advancement through both E2F and p27 regulation, the contribution of these pathways to a given cell cycle arrest is poorly understood.

Much of the molecular understanding of pRb function has been obtained through the use of gene-targeted mouse models to selectively disrupt elements of the G_1 checkpoint. Genetic disruption of the mouse *Rb1* gene results in embryonic lethality between embryonic day (E) 13.5 and E15 (*12*) with severe defects in multiple organ systems. However, the major defects were found to be secondary to ectopic proliferation in the trophoblast cells of the placenta that limits the nutrient transport to the developing embryo (*13*). $Rb1^{-/-}$ animals rescued with a wild-type placenta were viable until birth at which time all animals died from a severe defect in skeletal muscle differentiation that prevented the neonatal animals from respiring (*13, 14*). $Rb1^{+/-}$ mice are viable but develop pituitary tumors at approximately one year of age (*15, 16*). Fibroblasts generated from $Rb1^{-/-}$ embryos have a shorter G1 phase of the cell cycle, with a coincident reduction in cell size (*17*), and are unable to respond to ectopic arrests induced by p16

(*18*) and TGFβ (*19*). Genetic disruption of p27 in *Cdkn1b*$^{-/-}$ mice results in hyperplasia in multiple organs, female sterility and a late onset of partially penetrant pituitary tumors after 1 year of age (*20*). Compound mutant *Rb1*$^{+/-}$ *Cdkn1b*$^{-/-}$ mice are viable but rapidly succumb to pituitary and thyroid tumors at an average of 178 days (*21*). Taken together the genetic disruption of *Rb1* has revealed a context dependent role for pRb in cell cycle regulation and tumor suppression. Furthermore, pRb and p27 appear to play an overlapping role in tumor suppression, however the basis by which this occurs is not well defined.

To investigate the relative contribution of E2F regulation to cell cycle control mediated by pRb, we generated a gene-targeted mouse model that selectively disrupts the interaction between pRb and E2F transcription factors. We find that fibroblasts generated from *Rb1*$^{\Delta G/\Delta G}$ embryos have deregulation of E2F activity yet maintain the ability to regulate proliferation in multiple cellular contexts. To investigate pathways that might compensate for loss of E2F binding in the ΔG-mice we inter-crossed our mice with *E2f1*$^{-/-}$ and *Cdkn1b*$^{-/-}$ mice. Combination of E2F1 loss with the ΔG mutation does not alter proliferative control. In contrast, combined disruption of the p27 gene *Cdkn1b* with the ΔG mutation results in proliferative defects in cell culture and the compound mutant mice develop a high incidence of pituitary tumors. Taken together this work suggests that E2F regulation by pRb is dispensable for cell cycle control as p27 accumulation can function to maintain appropriate cell cycle control in multiple contexts and function as a barrier for tumorigenesis.

4.3 Methods

4.3.1 Gene targeting and cell culture

ES cell culture, transfection and selection was preformed by the Van Andel Institute Germline modification facility (Grand Rapids, MI). ES cells correctly targeted by the ΔG-pRb targeting vector shown in figure 4.1a were indentified by Southern Blot using probes outside the 5' and 3' ends of homology to ensure proper integration at the *Rb1* locus. A probe specific to the Neomycin resistance gene was also used to ensure that targeted clones only contained a single site of integration of the targeting vector. ES clones were then injected into blastocysts to generate chimeric mice. Male chimeras were mated with *EIIa-cre* transgenic mice to remove the PGK-Neo selectable marker that was flanked by Loxp sites. Progeny were then intercrossed to generate mice that had excised the selectable maker and did not express the Cre-recombinase. The mice were genotyped by amplification of a short sequence that surrounds the remaining LoxP site. Using L-F (ctgcaatctgcgcatttta) and L-R (cgatgctgcaggcctataat) a 250 bp and a 330 bp fragment was produced that corresponds to the mutant and wild-type allele respectively. Wild-type and $Rb1^{\Delta G/\Delta G}$ fibroblasts were derived from matched littermates and experiments were carried out using passage 3-5 MEFs. Asynchronous cell populations were cultured according to standard methods. Cell culture was carried out in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2mM L-glutamine, penicillin (50U/mL) and streptomycin (50μg/mL). Cells deprived of serum were cultured for 72 hours in media with 0.1% FBS. Confluence arrested cells were cultured for 7 days after reaching confluence in 10% FBS. All animals were housed and handled as approved by the

Canadian Council on Animal Care. Mice were monitored throughout their lives and animals were euthanized after the development of signs of tumor burden. Euthanized animals were subjected to a necropsy with abnormal tissues and tumors fixed in formalin and processed for histological assessment. Sections of tumors and tissues fixed in formalin for at least 72 hours were washed for 3 days in PBS then transferred to 70% ethanol. The tissues were embedded in paraffin and five μm sections were cut from superficial and deep sections of the blocks. Sections were subsequently stained with Hematoxylin and eosin and images were captured on a Zeiss Axioskop40 microscope and Spot Flex camera using EyeImage software (Empix Imaging, Mississauga, Ontario, Canada).

4.3.2 Retroviral infections

Retroviral infections were performed as described in Pear et al, 1993 (*22*). The BOSC packing cells were plated at a density of ten million cells per 15cm plate the day before the transfections. The following the day the cells were transfected with 60μg of pBabe plasmid or pBabe containing p16 or p27 using calcium phosphate and the next morning the media was replaced. The media was removed 48 hours later, filtered through a 0.45μm filter and supplemented with 4μg/mL of Polybrene. The filtered viral supernatant was placed directly on MEFs that had been plated the previous day at 8 x 10^5 cells in a 10cm dish. Fresh media was added to the transfected BOSC cells for another 12 hours. After 12 hours the media from the MEFs was removed and a second round of infection was preformed by once again adding the filtered viral supernatant with Polybrene to the MEFs. The viral supernatant was incubated on the MEFS for a further 8-

12 hours and then replaced with media containing 5μg/mL of puromycin for 4 days. The infected MEFs were then replated at low density in puromycin containing media and labeled with BrdU for subsequent flow cytometry analysis.

4.3.3 Protein interaction analysis and western blotting

To generate extracts the cells were washed twice with phosphate buffered saline (PBS) and collected into 1mL of Gel Shift Extract (GSE) buffer (20mM Tris pH7.5, 420mMNaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, 5mg/mL leupeptin, 5mg/mL aprotinin, 0.1mM Na_3VO_4, 0.5mM NaF and 1mM DTT). Extracts were frozen at -80°C. Extracts were thawed and cellular debris were removed by centrifugation at 14,000 rpm. For immunoprecipitations extract was diluted in IP Wash buffer (20mM Tris pH 7.5, 200mM NaCl, 1.5mMMgCl_2, 0.2mM EDTA, 25mM DTT and 0.1% NP-40). pRb complexes were immunoprecipitated with C-18 (Santa Cruz) for E2F3, bound to protein G-sepharose (GE healthcare). Immunoprecipitations were incubated with rocking for 1 hour at 4°C. The protein G-sepharose beads were washed twice with IP wash buffer then resuspended in 1X-SDS-PAGE sample buffer and boiled at 95°C for 5 min to elute the bound proteins. The eluted material was resolved by electrophoresis on a sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-8%PAGE) gel. Proteins were transferred to a nitrocellulose membrane by standard techniques. E2F1 was detected by KH20 (Santa Cruz) and E2F3 by PG37 (Upstate) and pRb by G3-245 (BD Pharmingen).

Purified GST, GST-E7 and GST-E1A were obtained as a kind gift from Biljana Todorovic and Joe Mymryk. Two micrograms of the GST-fusion proteins was diluted in low salt GSE buffer (20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1.5 mM MgCl2, 0.2 mM

EDTA, 1 mM DTT and 0.1 % Nonidet P40) and incubated with whole cell extract from *Rb1*$^{+/+}$ or *Rb1*$^{\Delta G/\Delta G}$ fibroblasts. The GST complexes were precipitated with glutathione–Sepharose and washed twice with low salt GSE buffer and eluted with 1 × SDS/PAGE sample buffer. Samples were electrophoresed on SDS/PAGE (8% gel) and blotted using the same antibodies outlined for the immunoprecipitation experiments above and input levels of recombinant proteins was detected by coomassie stain.

4.3.4 Electromobility shift assays

Electromobility shift assays were performed using DNA probes described in Hurford et al. (*23*). These probes were labeled with 50μCi of [α-^{32}P]dCTP with klenow fragment for 15 min at room temperature. The labeled probes were purified on a G25 spin column. Extract was prepared from confluent MEFs as described above. Each sample was diluted in EMSA buffer (20mM Tris pH7.5, 4% Ficoll 400-DL (Sigma), 2.5mM $MgCl_2$, 40mM KCl, 0.1mM EGTA, 2mM spermine, 0.5mM DTT, 0.25μg salmon sperm DNA, 10μg bovine serum albumin) and 5μg of nuclear extract. Samples with cold competitors were first incubated with 40ng of wild-type or mutant unlabelled oligonucleotides for 10 min on ice. 400pg of labeled probe was then added to each reaction and incubated on ice for 10 min. For antibody supershifts antibodies were added and the samples were incubated on ice for a further 25 min. For supershifts 1μg of the following antibodies were used; pRb 21C9 (a kind gift from Sibylle Mittnacht), CDK2 (Upstate), CDK4 C-22 (Santa Cruz Biotechnology), Cyclin E M-20 (Santa Cruz Biotechnology), p107 C-18 (Santa Cruz Biotechnology) and p130 C-20 (Santa Cruz Biotechnology). Samples were loaded onto a 4% polyacrylamide gel (containing 0.25X

Tris-borate-EDTA and 2.5% glycerol) and electrophoresed at 4°C for 4 h at 180V. Gels were dried and complexes were detected by autoradiography.

4.3.5 RNA quantification

Expression levels of the E2F target genes, *Pcna, Ccne1, Ccna2, Tyms* and *Rbl1* were determined using the Quantigene Plex 2.0 reagent system from Affymetrix (Santa Clara, CA) and a BioPlex200 multiplex analysis system according to manufacturers instructions. Expression levels were quantified were normalized to the expression of acidic ribosomal phosphoprotein P0 (*Rblp0*).

4.3.6 Cell Cycle analysis

Cell cycle analysis of MEFs was performed by pulse-labeling cells with bromodeoxyuridine (BrdU) (Amersham Biosciences) according to manufacturers instructions 1.5 hours before harvesting cells. The cells were fixed in ethanol and immunostained with anti-BrdU antibodies (BD Biosciences), along with propidum iodide as reported in Classon et al. (*24*). Cell populations were analyzed by flow cytometry on a Beckman-Coulter EPICS XL-MCL instrument.

4.3.7 Immunohistochemistry

To quantify intestinal proliferation three age-matched $Rb1^{+/+}$and $Rb1^{\Delta G/\Delta G}$ pairs were injected with 200μL of 16μg/mL BrdU (sigma) 1 hour before sacrifice. Intestines were then isolated fixed in formalin, embedded and sectioned according to standard protocols. To quantify proliferation in the retina of embryos $Rb1^{\Delta G/+}Cdkn1b^{+/-}$ females were

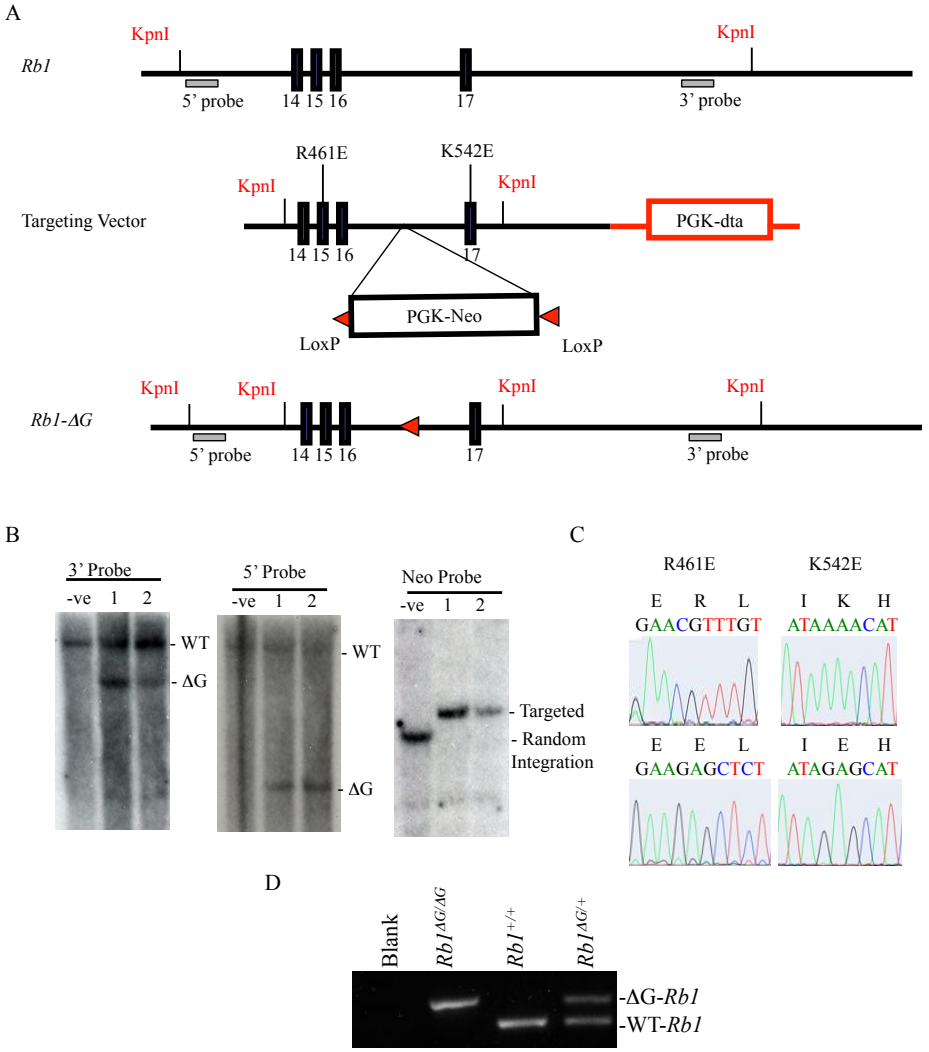
injected with 200μL of 16μg/mL BrdU on the 15th day of pregnancy 2 hours before sacrifice. Embryos were then isolated, genotyped and fixed in formalin. The heads of the embryos were embedded and sectioned until sections of the retina were obtained. BrdU incorporation was detection was performed on paraffin sections that had been deparaffinized and rehydrated using a series of xylene and ethanol washes. The sections were brought to a boil in sodium citrate buffer and then maintained at 95°C for 10 min. The cooled sections were rinsed in water three times for 5 minutes each time and then rinsed in PBS for 5 minutes. The sections were blocking solution (Phosphate buffered saline (PBS) supplemented with 2.5% horse serum and 0.3% Triton X) for 1 hour. The sections were incubated with anti-BrdU (BD-Biosciences) in blocking buffer overnight at 4°C and then rinsed in PBS three times for 5 minutes each time. The slides were incubated with horse anti-mouse immunoglobulin G-fluorescein isothiocyanate (Vector) for 1h and then rinsed in PBS. The slides were mounted with Vectashield plus DAPI (Vector) and sealed with nail polish. Fluorescent images were captured on a Zeiss Axioskop40 microscope and Spot Flex camera and colored using EyeImage software (Empix Imaging, Mississauga, Ontario, Canada).

4.4 Results

4.4.1 Targeted disruption of E2F binding in the mouse *Rb1* gene

To disrupt the interaction between pRb and the transactivation domain of E2F transcription factors, two substitutions (R461E and K542E) were introduced into the mouse *Rb1* gene. These substitutions are the mouse equivalents to R467E and K548E described in chapter 3 as the ΔGn mutant. For simplicity mouse pRb with R461E and

K542E substitutions is now defined as ΔG-pRb. As shown in the genomic structure of the mouse *Rb1* gene (Fig 4.1a), R461 and K542 are found in exons 15 and 17 respectively. A 1.5 kb intron is found between exon 16 and 17 that was utilized to insert the selectable marker. A gene-targeting vector was constructed with 11kb of homologous sequence containing the PGK-Neo selectable marker cassette inserted 4kb from the end of the 5' end of homology as depicted in figure 4.1a. The substitutions were introduced into the exons 15 and 17 along with novel KpnI sites upstream of the R461E substitution and downstream of the K542E substitution. The PGK-dta cassette was inserted outside the region of homology to select against clones that contained random integration of the vector. After selection of embryonic stem cells for G418 resistance, DNA from 471 clones was screened for correct integration of the targeting cassette. As depicted in figure 4.1a, the WT-*Rb1* gene has a single 23kb KpnI fragment that can be detected by southern blot with either a 5' or 3' prime probe. Correct integration of the targeting vector on the 5' and 3' side of the PGK-NEO cassette results in the introduction of two novel KpnI sites to produce 6kb and 13kb fragments detected by the 5' and 3' probes respectively. As shown in figure 4.1b, two clones were identified to have correctly targeted the mouse *Rb1* gene with the introduced KpnI sites on one of the alleles (Fig. 4.1b). A probe specific to the PGK-Neo cassette was also used to confirm that there was only a single integration site in the clones used (Fig. 4.1b).



A) Targeting scheme to introduce the R461E and K542E into exons 15 and 17 of the mouse *Rb1*. The targeting vector with 11kb of homology with the LoxP flanked PGK-Neo inserted into the intron 16 and PKG-dta outside of the homology is shown. The targeted allele is also shown with the introduced KpnI sites that were utilized for screening clones for proper integration. (B) Southern blot analysis of genomic DNA from ES clones selected for G418 resistance. The correct size of wild-type and targeted ΔG KpnI fragments are indicated for both the 3', 5' probes and the size of the fragment detected by the neo probe. (C) Sequencing of DNA from embryos homozygous for the ΔG allele shows the introduced substituties R461E and K542E (D) PCR genotyping of the ΔG-mutation using primers that flank the remaining LoxP site to produce a 330bp and 250bp band for the ΔG and WT- alleles respectively.

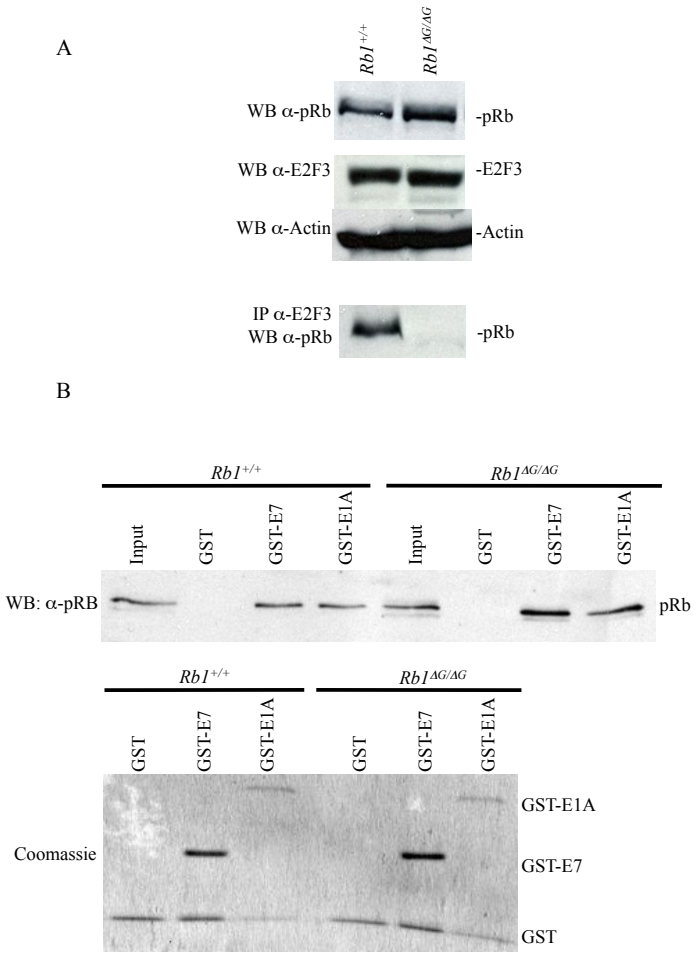
Figure 4-1 Targeting the mouse *Rb1* gene

The targeted embryonic stem cells were used to generate chimeric animals that were subsequently bred to mice expressing the Cre recombinase in order to remove the PGK-Neo cassette and leave only a single LoxP site within the intron downstream of exon 16. The mice were interbred and the substitutions were confirmed by direct sequencing of DNA isolated from embryos homozygous for the ΔG-allele (Fig. 4.1c). Genotyping of the ΔG-mice was accomplished by PCR using a pair of primers that flank the integrated LoxP site to produce an 80bp larger band for the ΔG allele due to the integrated LoxP site and surrounding sequence (Fig. 4.1d).

To assess the disruption of pRb-E2F interaction extract was generated from asynchronously grown $Rb1^{+/+}$ and $Rb1^{\Delta G/\Delta G}$ fibroblasts. As shown in figure 4.2a, pRb is expressed at similar levels in both the $Rb1^{+/+}$ and $Rb1^{\Delta G/\Delta G}$ fibroblasts, indicating that the substitutions do not disrupt the overall stability or expression of pRb. E2F3 was then immunoprecipitated from these extracts and immunoblotted for bound pRb. As shown in figure 4.2a, complexes between pRb and E2F3 are easily detected in the $Rb1^{+/+}$ cells; however, no complexes were observed between ΔG-pRb and E2F3 in the $Rb1^{\Delta G/\Delta G}$ cells. This suggests that the interaction defect in the gene-targeted mice is consistent with the initial *in vitro* studies. To confirm that other structural aspects of pRb were maintained in the $Rb1^{\Delta G/\Delta G}$ fibroblasts the interaction with the viral oncoproteins was assessed. There is a well-characterized interaction between the viral oncoproteins E7 and E1A with the LXCXE binding cleft of pRb. As shown in figure 4.2b, GST-E7 and GST-E1A are able to form stable complexes with pRb from extract generated from both $Rb1^{+/+}$ and $Rb1^{\Delta G/\Delta G}$ fibroblasts, confirming that the LXCXE binding cleft is maintained in ΔG-pRb. Taken together the introduction of the R461E and K548E substitutions into the mouse *Rb1* gene

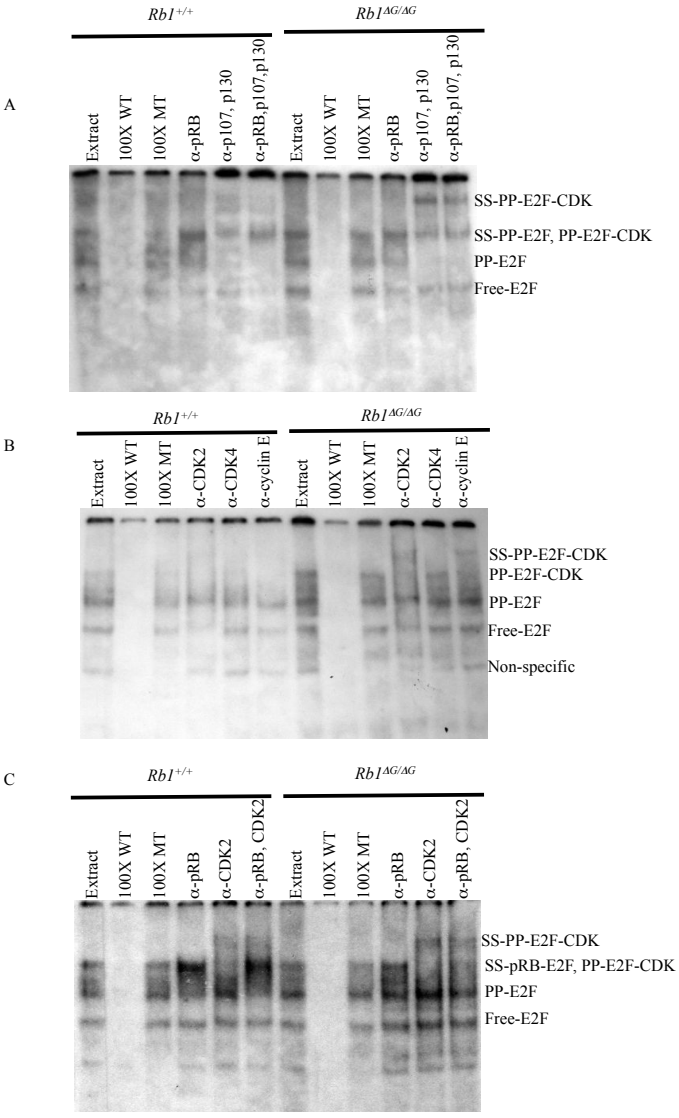
produces a discrete defect in the interaction with E2F transcription factors but preserves the overall stability and interaction with proteins at the LXCXE binding cleft.

To further characterize the interaction defects in the *Rb1*$^{\Delta G/\Delta G}$ mice we analyzed E2F complexes using an electromobility shift assay (EMSA) with a oligonucleotide derived from the adenovirus E2 promoter (*23*). In fibroblasts, three distinct types of E2F complexes are detected bound to the E2F probe. The fastest migrating band corresponds to free E2F/DP complexes (Fig 4.3). The next band consists of the pocket proteins (pRb, p107 or p130) bound to E2F/DP, while the shortest migrating complex corresponds to Cyclin/CDK bound to pocket protein-E2F complexes. Importantly, the complexes with Cyclin/CDK proteins consist only of p107 and p130 and are mediated by a high affinity cyclin binding site that is absent in pRb (*25*). As shown in figure 4.3a, there appears to be an increase in the relative amount of pocket protein-E2F/DP-Cyclin/CDK complexes in *Rb1*$^{\Delta G/\Delta G}$ fibroblasts in contrast to wild-type cells. This suggests that the balance of E2F complexes is shifted towards binding to p107 and p130 with the corresponding complexes with CDKs in the *Rb1*$^{\Delta G/\Delta G}$ cells. To further delineate the composition of pocket protein complexes a pRb- specific antibody was utilized to shift pRb-E2F complexes. Addition of the pRb specific antibody to *Rb1*$^{+/+}$ fibroblasts produces a new band that migrates similar to the Pocket-protein-Cyclin/CDK complexes (Fig. 4.3a). In extracts derived from *Rb1*$^{\Delta G/\Delta G}$ fibroblasts, addition of the pRb specific antibody does not alter the distribution of E2F complexes, suggesting that ΔG-pRb is unable to interact with E2F proteins. Furthermore, addition of p107 and p130 antibodies was sufficient to alter the migration of the majority of the pocket protein-E2F complexes in *Rb1*$^{\Delta G/\Delta G}$ extract (Fig. 4.3a).



(A) Immunoprecipitation of E2F3 complexes from *Rb1*$^{+/+}$ and *Rb1*$^{\Delta G/\Delta G}$ fibroblasts. Input levels of pRb, E2F3, actin and the amount of bound pRb co-precipitaed with E2F3 is shown as detected by western blotting. (B) Recombinant GST, GST-E7 and GST-E1A were incubated with from *Rb1*$^{+/+}$ and *Rb1*$^{\Delta G/\Delta G}$ fibroblasts and precipitated using glutathione–Sepharose. The relative amount of input and bound pRB was detected by Western blotting and the amount of input GST proteins was detected by coomassie.

Figure 4-2 The ΔG mutation selectively disrupts the interaction with E2F transcription factors



Electromobility shift assay (EMSA) were utilized to analyze complexes bound to a radiolabelled fragment derived from the adenovirus E2 promoter from extract generated from both *Rb1*$^{+/+}$ and *Rb1*$^{\Delta G/\Delta G}$ fibroblasts. The distinct E2F complexes are indicated as Free-E2F/DP complexes, pocket proteins bound to E2F/DP (PP-E2F) and pocket proteins bound to E2F/DP associated with cyclin/CDK complexes (PP-E2F-CDK). The composition of specific complexes was determined by the additions of antibodies to shift the migration and these are indicated with the SS-prefix. (A) The addition of antibodies to identify the distinct pocket protein complexes, (B) antibodies were added to characterize the complexes with CDK/Cyclins and (C) antibodies were added to detect the presence of pRB-E2F complexes in *Rb1*$^{+/+}$ and *Rb1*$^{\Delta G/\Delta G}$ extract.

Figure 4-3 The ΔG mutation disrupts the formation of pRb-E2F complexes

To confirm the composition of the complexes observed in the *Rb1*$^{\Delta G/\Delta G}$ fibroblasts, antibodies directed against cyclins and CDKs were utilized. As shown in figure 4.3b, addition of antibodies to CDK2 and Cyclin E, but not to CDK4, shifts the pocket protein-Cyclin/CDK-E2F/DP complexes. This suggests that in the *Rb1*$^{\Delta G/\Delta G}$ cells, there are significantly more complexes between pocket proteins bound CDK2/Cyclin E proteins. Since the shifted pRb-E2F complexes migrate similarly to the complexes with bound CDK2/Cyclin E, we first shifted the CDK2/Cyclin E complexes using a CDK2 antibody, then added a pRb antibody to identify pRb-E2F complexes. As shown in figure 4.3c, extract generated from *Rb1*$^{+/+}$ has a significant amount of pRb-E2F complexes, however no pRb-E2F complexes were identified from the ΔG- fibroblasts. Taken together, this further confirms the defect in E2F binding produced by the ΔG mutation. Specifically the ΔG mutation disrupts complex formation between pRb and E2F transcription factors leading to increased prevalence of p107 and p130 complexes and an increase in complexes with CDK2/Cyclin E.

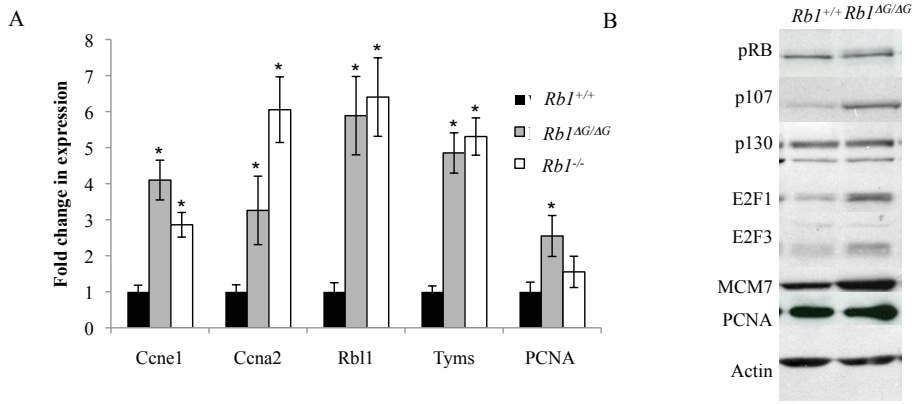
Given the disruption of pRB-E2F complexes in the *Rb1*$^{\Delta G/\Delta G}$, we next sought to investigate the regulation of E2F target gene expression in the *Rb1*$^{\Delta G/\Delta G}$ fibroblasts. As shown in figure 4.4a, the RNA levels of five canonical E2F target genes are significantly increased in the serum starved *Rb1*$^{\Delta G/\Delta G}$ cells. Furthermore, the deregulation of E2F targets is similar to *Rb1*$^{-/-}$ cells, suggesting that *Rb1*$^{\Delta G/\Delta G}$ fibroblasts are completely defective for regulating E2F target gene expression. E2F target genes were observed to be upregulated at the protein level as well in ΔG-fibroblasts as shown in figure 4.4b. This

fits with the inability of ΔG-pRb to interact with E2F3 and the lack of ΔG-pRb-E2F complexes capable of binding the labeled E2F probe.

4.4.2 *Rb1*$^{\Delta G/\Delta G}$ mice maintain proliferative control

Given the deregulation of E2F target genes in the *Rb1*$^{\Delta G/\Delta G}$ fibroblasts, current understanding of cell cycle progression would predict that the *Rb1*$^{\Delta G/\Delta G}$ fibroblasts would display altered cell cycle control in a similar manner as the *Rb1*$^{-/-}$ cells. Shown in figure 4.5a-b, the *Rb1*$^{-/-}$ cells have an increased proportion of cells in S-phase in both asynchronous (Fig 4.5a) and in serum starved (Fig. 4.5b) cell populations. Strikingly, the *Rb1*$^{\Delta G/\Delta G}$ cells appear to maintain cell cycle control and have similar cell cycle distributions as *Rb1*$^{+/+}$ cells (Fig. 4.5a,b). Thereby, despite deregulated E2F target gene expression the *Rb1*$^{\Delta G/\Delta G}$ cells are capable of maintaining the G_1 checkpoint and prevent the ectopic S phase entry that is observed in *Rb1*$^{-/-}$ cells.

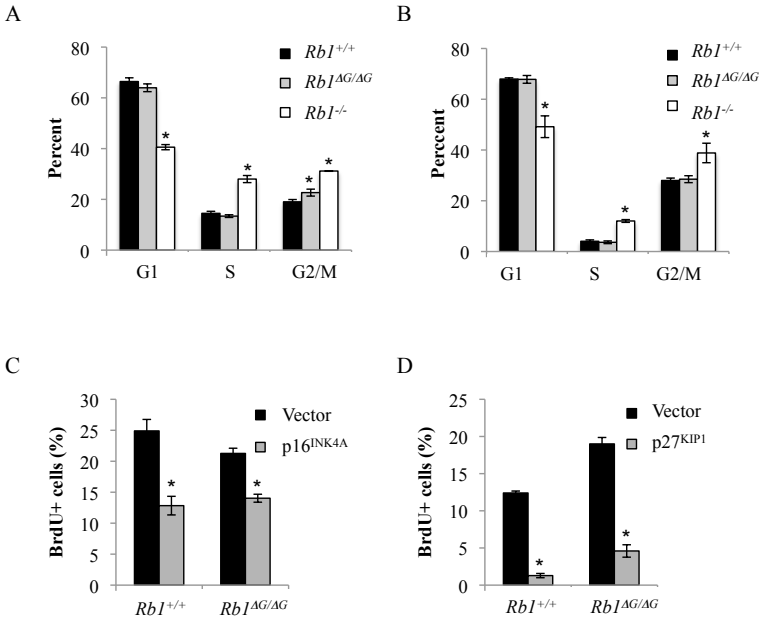
To investigate cell cycle control in other cellular contexts, the *Rb1*$^{+/+}$ and *Rb1*$^{\Delta G/\Delta G}$ fibroblasts were virally transduced with p16^{INK4A} or p27^{KIP1} to induce an acute arrest of asynchronous cells. In a similar manner to serum starvation, the *Rb1*$^{\Delta G/\Delta G}$ fibroblasts respond to the ectopic expression of p16^{INK4A} or p27^{KIP1} and arrest to a similar extent as *Rb1*$^{+/+}$ cells. In contrast *Rb1*$^{-/-}$ cells have a well-described defect in their ability to respond to the expression of ectopic CDK inhibitors (*18*). The ΔG-pRb mutation causes a clear defect in the interaction with E2F transcription factors that results in the deregulation of E2F target genes, but this does not translate into discrete cell cycle defects in *Rb1*$^{\Delta G/\Delta G}$ fibroblasts, as other mechanisms appear to exist to prevent ectopic S-phase entry.



(A) Relative expression of mRNA levels of E2F target gene transcripts in serum starved $Rb1^{\Delta G/\Delta G}$ and $Rb1^{-/-}$ fibroblasts to wild-type cells. Data is normalized to the expression of acidic ribosomal phosphoprotein P0 (Rblp0) and error bars indicated one standard deviation from the mean. The asterisks indicate a statistically significant difference (Students T-test; $P<0.05$) (B) The protein level of a series of E2F target genes are shown in extract generated from serum starved $Rb1^{+/+}$ and $Rb1^{\Delta G/\Delta G}$ fibroblasts as detected by western blotting with actin as a control for loading.

Figure 4-4 Deregulation of E2F target gene expression in $Rb1^{\Delta G/\Delta G}$ fibroblasts

In addition to the cell cycle defects observed in *Rb1*$^{-/-}$ fibroblasts, the mice also have severe defects in development that leads to embryonic lethality around E13.5. We therefore investigated the development of the *Rb1*$^{\Delta G/\Delta G}$ to characterize the role of E2F regulation in multiple developmental contexts. Despite the deregulated E2F target gene expression observed in cell culture, live *Rb1*$^{\Delta G/\Delta G}$ mice were obtained after birth (Fig. 4.6a). Viable *Rb1*$^{\Delta G/\Delta G}$ mice have been observed into adulthood that are indistinguishable from wild-type litter-mates (Fig. 4.6b). We next isolated embryos at distinct developmental stages to characterize the viability of the *Rb1*$^{\Delta G/\Delta G}$ mice. As shown in figure 4.6c, *Rb1*$^{\Delta G/\Delta G}$ embryos were obtained at expected Mendelian ratios up until birth. Targeted disruption of pRB in the intestine leads to inappropriate proliferation of the differentiated villi of the epithelium (*26*). We thus investigated the incorporation of BrdU into the intestinal epithelium of adult *Rb1*$^{\Delta G/\Delta G}$ mice. As shown in figure 4.6d and quantified in figure 4.6e, there appears to be no ectopic cell cycle entry in the villi of *Rb1*$^{\Delta G/\Delta G}$ mice or altered proliferation of the crypt cells. This suggests, along with the viability of *Rb1*$^{\Delta G/\Delta G}$ animals, that proliferative control is largely maintained in the *Rb1*$^{\Delta G/\Delta G}$ mice.

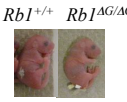


(A-B) Cell cycle distributions of fibroblasts of indicated genotypes as determined by BrdU incorporation and DNA content detected by flow cytometry. (A) Cell cycle distributions of asynchronous populations of fibroblasts and (B) cell cycle distribution of serum starved cells. (C-D) *Rb1*$^{+/+}$ or *Rb1*$^{\Delta G/\Delta G}$ fibroblasts were infected with retrovirus expressing an empty pBABE vector control, p16 (C) or p27(D). After selection of infected cells with puromycin cells were pulse labeled with BrdU and incorporation was measured by flow cytometry with the percent incorporation shown. (A-D) All bars represent the mean of three independent samples and error bars represent one standard deviation from the mean. The asterisks indicate a statistically significant difference (Students T-test; $P<0.05$)

Figure 4-5 The *Rb1*$^{\Delta G/\Delta G}$ fibroblasts maintain proliferative control.

However, approximately half of the neonatal *Rb1*$^{\Delta G/\Delta G}$ mice were found dead shortly after birth (Fig. 4.6c) and some neonatal mice were observed in severe respiratory distress. Given that *Rb1*$^{-/-}$ mice with rescued placenta also die shortly after birth we wanted to investigate if a similar phenotype was occurring in the *Rb1*$^{\Delta G/\Delta G}$ mice. *Rb1*$^{-/-}$ embryos have a defects in skeletal muscle development and their diaphragms that lead to severe respiratory compromise (*14*). In the *Rb1*$^{\Delta G/\Delta G}$ animals there also appears to be a defect in their diaphragm development that is characterized by inappropriate differentiation and cell death (Fig. 4.6f). Furthermore, neonatal *Rb1*$^{\Delta G/\Delta G}$ mice appear to have a thickened and abnormal development of the epithelial lining of the lung, as shown in figure 4.6f, that resemble the neuroendocrine hyperplasia reported in lungs of chimeric *Rb1*$^{-/-}$ animals (*27*). It is not clear how the defects in muscle and lung development contribute to the neonatal lethality of a fraction of *Rb1*$^{\Delta G/\Delta G}$ animals. The survival of *Rb1*$^{\Delta G/\Delta G}$ at expected ratios until birth and survival of some animals suggests that the severe proliferative defects in the placenta that lead to the embryonic lethality of *Rb1*$^{-/-}$ mice are not occurring in the *Rb1*$^{\Delta G/\Delta G}$ mice. Nevertheless, the lung and muscle abnormalities suggest that in some contexts deregulated E2F activity is sufficient to lead to partially penetrant developmental dysfunction that warrants further investigation.

A



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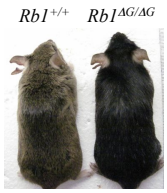
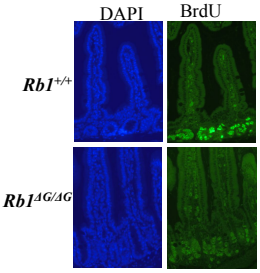


C

Genotype	E13.5	E18.5	P0	P14
$Rb1^{+/+}$	**11** (16)	**24** (23)	15 (15)	**163** (127)
$Rb1^{\Delta G/+}$	**34** (32)	**46** (46)	29 (31)	**291** (254)
$Rb1^{\Delta G/\Delta G}$	**19** (16)	**22** (23)	**9 Live** *9 Dead* (15)	**54** (127)
Total	64	92	61	508

D



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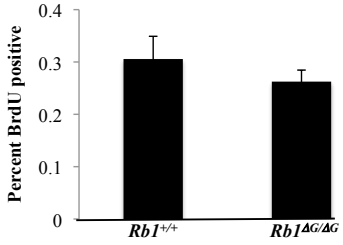
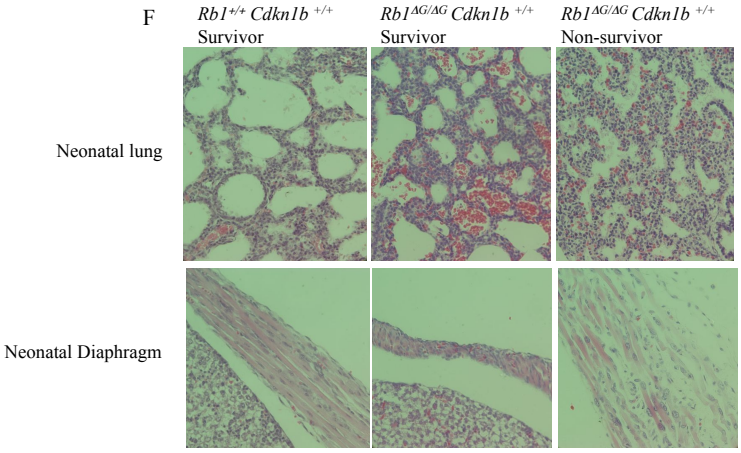


F



Photograph of P0.5 neonatal (A) and adult (B) $Rb1^{+/+}$ and $Rb1^{\Delta G/\Delta G}$ mice. (C) The genotypes of embryos and animals generated from a $Rb1^{\Delta G/+}$ intercross at various stages of development. The number of observed embryos/animals is shown in bold font and the expected number is displayed in parentheses. The number of dead $Rb1^{\Delta G/\Delta G}$ neonatal mice found shortly after birth is indicated in italics. (D) The incorporation of BrdU in the intestines of adult $Rb1^{+/+}$ and $Rb1^{\Delta G/\Delta G}$ is shown as detected by immunofluorescence. (E) The percentage of BrdU positive DAPI stained nuclei is quantified from three pairs of $Rb1^{+/+}$ and $Rb1^{\Delta G/\Delta G}$ animals with error pars indicating one standard deviation from the mean. (F) Hematoxylin and eosin staining of sagittal sections from P0.5 neonatal animals of indicated genotypes showing the defects in lung and muscle development from surviving and non-surviving $Rb1^{\Delta G/\Delta G}$ animals.

Figure 4-6 The majority of $Rb1^{\Delta G/\Delta G}$ animals are viable with maintained proliferative control

4.4.3 p27 functions in an overlapping manner to control proliferation in the absence of E2F regulation by pRb

Given previous findings that pRb is capable of regulating distinct overlapping pathways to maintain proliferative control, we next sought to investigate the contribution of these pathways in the *Rb1*$^{\Delta G/\Delta G}$ mice. To disrupt the 'specific' interaction between pRb and E2F1 we crossed the *Rb1*$^{\Delta G/+}$ mice with E2f1$^{-/-}$ animals. pRb can also regulate the levels of p27 indirectly through the interaction with Cdh1 and Skp2 independently of E2Fs. To abrogate this pathway of cell cycle control we crossed the *Rb1*$^{\Delta G/+}$ mice with *Cdkn1b*$^{-/-}$ mice that lack the p27 protein. As shown in figure 4.7a, viable *Rb1*$^{\Delta G/\Delta G}$ *E2f1*$^{-/-}$ and *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ were obtained at less than expected frequencies but consistent with neonatal lethality of the *Rb1*$^{\Delta G/\Delta G}$ animals. However, as shown in figure 4.8a, *Rb1*$^{\Delta G/\Delta G}$ *E2f1*$^{-/-}$ fibroblasts maintain proliferative control in response to serum starvation. This suggests that mechanisms exist beyond E2F regulation to control proliferation as the introduction of the ΔG mutation into *E2f1*$^{-/-}$ mice effectively disrupts both the general and specific E2F binding sites in pRB. In contrast, proliferative control is disrupted in the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ fibroblasts with increased cells in S-phase and G2/M suggesting a defective G_1 arrest of the cells (Fig 4.8a). This suggests that, at least in some contexts, the proliferative control in *Rb1*$^{\Delta G/\Delta G}$ cells is mediated by the activity of p27.

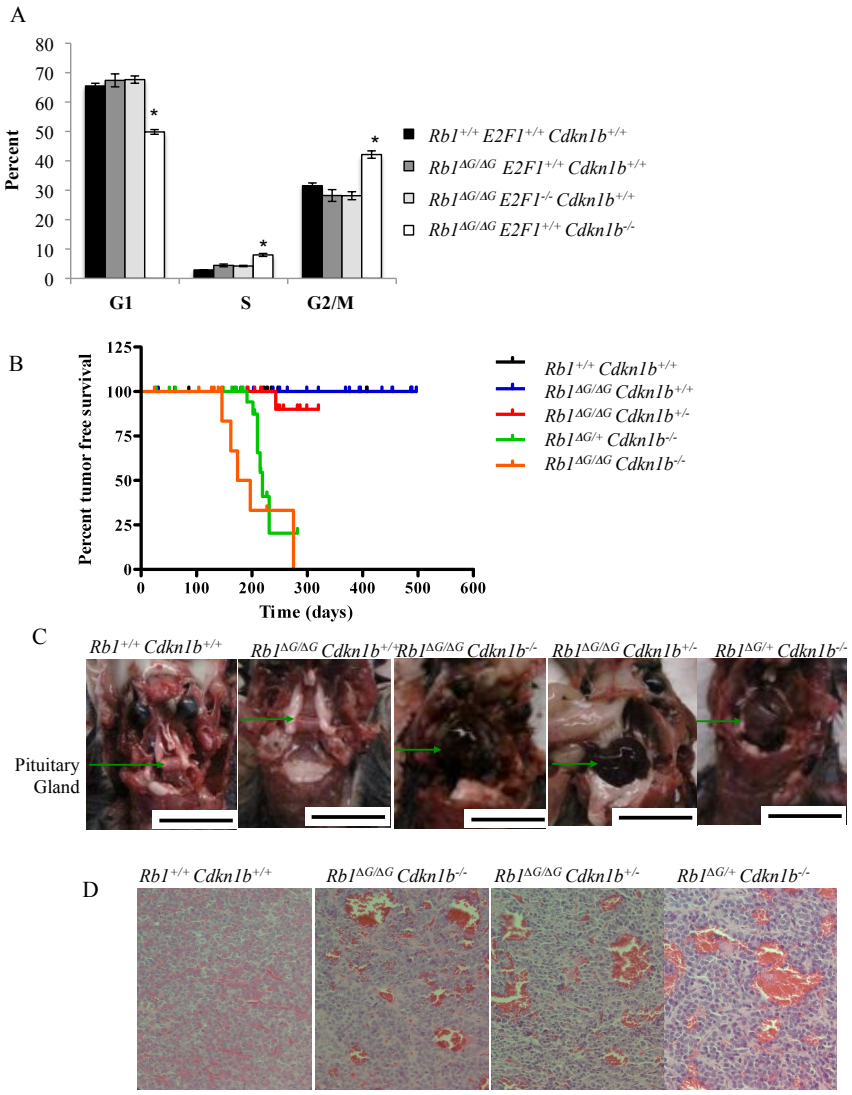
$Rb1^{\Delta G/+}$; $E2F1^{+/-}$ X $Rb1^{\Delta G/+}$; $E2F1^{+/-}$		$Rb1^{\Delta G/+}$; $Cdkn1b^{+/-}$ X $Rb1^{\Delta G/+}$; $Cdkn1b^{+/-}$	
Genotype	**P14**	**Genotype**	**P14**
$Rb1^{+/+}$; $E2F1^{+/+}$	**19** (13)	$Rb1^{+/+}$; $Cdkn1b^{+/+}$	**25** (15)
$Rb1^{+/+}$; $E2F1^{+/-}$	**31** (25)	$Rb1^{+/+}$; $Cdkn1b^{+/-}$	**27** (29)
$Rb1^{+/+}$; $E2F1^{-/-}$	**16** (13)	$Rb1^{+/+}$; $Cdkn1b^{-/-}$	**12** (15)
$Rb1^{\Delta G/+}$; $E2F1^{+/+}$	**22** (25)	$Rb1^{\Delta G/+}$; $Cdkn1b^{+/+}$	**40** (29)
$Rb1^{\Delta G/+}$; $E2F1^{+/-}$	**59 (**51)	$Rb1^{\Delta G/+}$; $Cdkn1b^{+/-}$	**72** (59)
$Rb1^{\Delta G/+}$; $E2F1^{-/-}$	**35** (25)	$Rb1^{\Delta G/+}$; $Cdkn1b^{-/-}$	**22** (29)
$Rb1^{\Delta G/\Delta G}$;$E2F1^{+/+}$	**6** (13)	$Rb1^{\Delta G/\Delta G}$;$Cdkn1b^{+/+}$	**11** (15)
$Rb1^{\Delta G/\Delta G}$; $E2F1^{+/-}$	**12** (25)	$Rb1^{\Delta G/\Delta G}$; $Cdkn1b^{+/-}$	**19** (30)
$Rb1^{\Delta G/\Delta G}$; $E2F1^{-/-}$	**4** (13)	$Rb1^{\Delta G/\Delta G}$; $Cdkn1b^{-/-}$	**8** (15)
Total	204	Total	236

The genotypes of P14 offspring generated from a $Rb1^{\Delta G/+}E2F1^{+/-}$ or $Rb1^{\Delta G/+}Cdkn1b^{+/-}$ intercrosses is shown. The number of observed animals is shown in bold font and the expected number is displayed in parentheses.

Figure 4-7 Combination of the ΔG mutation with E2F1 or p27 deficiency does not alter the viability of $Rb1^{\Delta G/\Delta G}$ mice

We have followed the *Rb1*$^{\Delta G/\Delta G}$ mice to investigate tumor formation in these animals and have yet to identify animals with any morbidity or tumors with the oldest *Rb1*$^{\Delta G/\Delta G}$ over 1.5 years of age. In contrast *Rb1*$^{+/-}$ animals develop completely penetrant pituitary tumors within the first year of life (*28*). *Cdkn1b*$^{-/-}$ mice develop pituitary and thyroid tumors, but with a low penetrance and typically later in life after one year of age (*20*). However, the incidence of tumors is significantly increased when combined with *Rb1* disruption with a mean age of death 178 days for *Rb1*$^{+/-}$ *Cdkn1b*$^{-/-}$ animals (*21*). Given that the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ compound mutant fibroblasts displayed a defect in proliferative control we next sought to investigate tumor formation in animals carrying the ΔG-mutation along with p27 deficiency. As shown in figure 4.8b, the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ appear to rapidly develop pituitary tumors with similar kinetics as *Rb1*$^{+/-}$ *Cdkn1b*$^{-/-}$ animals, with the mean tumor free survival of these animals at 197 days (Fig. 4.8b). Furthermore, mice with *Rb1*$^{\Delta G/+}$ *Cdkn1b*$^{-/-}$ and *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{+/-}$ genotypes also develop pituitary tumors. Importantly, no *Rb1*$^{\Delta G/\Delta G}$ E2f1$^{-/-}$ animals have developed tumors or other signs of morbidity suggesting that the 'general site' and p27 regulation have a critical synergistic role in blocking pituitary tumorigenesis.

As shown in figure 4.8c, the pituitary glands of *Rb1*$^{+/+}$ and *Rb1*$^{\Delta G/\Delta G}$ are small structures located at the base of the brain that contain a regular arrangement of cells with a well organized vasculature (fig. 4.8d). In stark contrast, the pituitary tumors isolated from the ΔG/p27 deficient animals are grossly enlarged hemorrhagic structures that



(A) Cell cycle distributions of serum starved fibroblasts of indicated genotypes as determined by BrdU incorporation and DNA content detected by flow cytometry. All bars represent the mean of three independent samples and error bars represent one standard deviation from the mean. The asterisks indicate a statistically significant difference (Students T-test; $P<0.05$) (B) Kaplan-Meier survival proportions shown for mice of indicated genotypes. A significant difference between the survival of the indicated genotypes was identified (Log-rank Test; $P<0.0001$) (C) Gross photographs of normal pituitary gland morphology in one-year-old $Rb1^{+/+}Cdkn1b^{+/+}$ and $Rb1^{\Delta G/\Delta G}Cdkn1b^{+/+}$ animals. Gross photographs of pituitary tumor morphology from mice of indicated genotypes. (D) Hematoxylin and eosin staining of axial sections from pituitary tumors of indicated genotypes and sections of control pituitary gland from a $Rb1^{+/+}Cdkn1b^{+/+}$mouse.

Figure 4-8 Disruption of E2F regulation and p27 deficiency results in altered proliferative control and pituitary formation

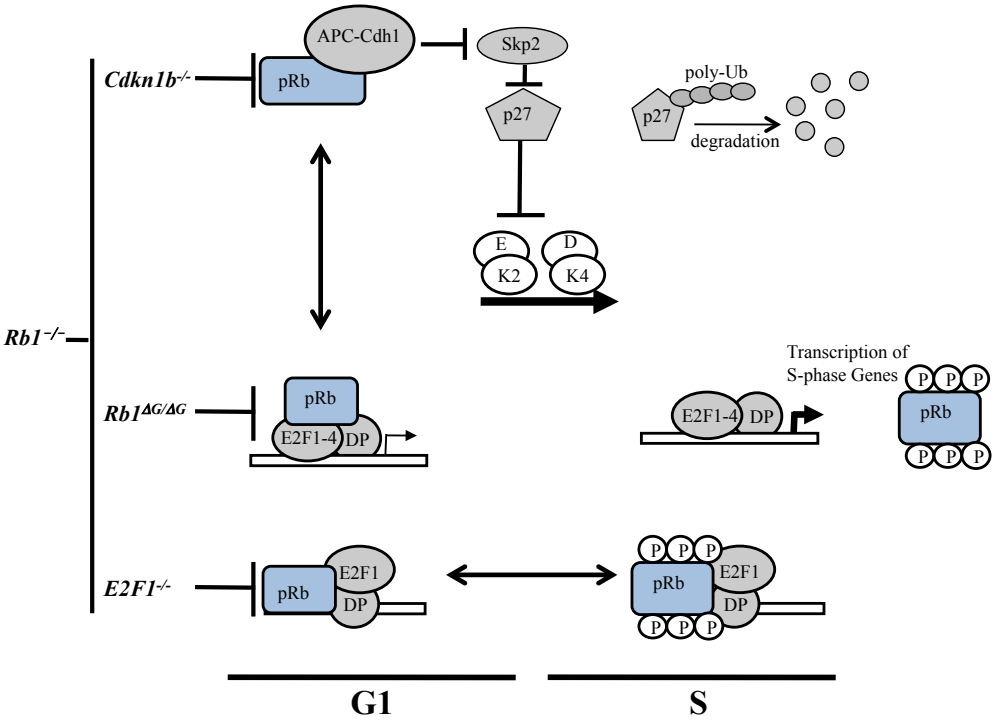
impinge upon the base of the brain (Fig 4.8c and d). The animals were observed to go through a rapid decline, characterized by significant weight loss and lethargy. Further, some animals displayed signs of neurological dysfunction including hemi-paralysis and an intracranial hemorrhage was observed upon necropsy of multiple animals.

Taken together these results suggest that deregulation of E2F signaling is not sufficient to allow ectopic proliferation and tumorigenesis in mice. The preliminary evidence presented here suggests that in the absence of E2F regulation by pRb, p27 functions to maintain proliferative control and block tumorigenesis. This is highlighted by the rapid development of tumors in mice that simultaneously disrupt both E2F regulation and p27. Thereby, pRb is capable of engaging multiple cellular pathways to prevent ectopic S-phase entry and provide a robust barrier to tumorigenesis.

4.5 Discussion

While current understanding typically highlights the role of pRb-E2F interactions in the regulation of proliferative control, our work highlights the importance of the multiple overlapping mechanisms of cell cycle arrest mediated by pRb. As shown in figure 4.9, pRb is capable of forming multiple interchangable complexes in the G_1 phase of the cell cycle to negatively regulate entry into S-phase. Specifically, pRb is capable of interacting with the transactivation domain of E2F transcription factors to restrict the activation of S-phase target genes. This regulation is mediated by the 'general' interaction between pRb and E2Fs and is disrupted by CDK phosphorylation. This site is abrogated by the ΔG mutation, which results in the deregulation of E2F target gene expression. Additionally the 'specific site' can form a unique interaction with E2F1

independently of the 'general site' and was disrupted by crossing the mice to E2f1$^{-/-}$ animals. Lastly pRb is capable of interacting with components of two ubiquitin ligase complexes Cdh1 and Skp2 to promote the degradation of Skp2 and protect p27 from degradation. This pathway was abrogated in *Cdkn1b*$^{-/-}$ as these animals do not express p27. As predicted from earlier work in chapter 3 the overall cell cycle control mediated by pRb is not regulated solely by E2F regulation of the 'general site', as other binding interfaces on pRb can function in the ΔG-mice to maintain proliferative control. We find that regulation of p27 has a critical function in maintaining proliferative control and tumor suppression, as combined disruption of the 'general site' along with p27 results in deregulated proliferation and tumorigenesis. In contrast, combined disruption of E2F1 and the 'general site' did not appear to alter proliferative control or tumor suppression. In contrast to *Rb1*$^{-/-}$ mice, the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ were viable and born at expected ratios. This suggests that the placental dysfunction that leads to the embryonic lethality of the *Rb1*$^{-/-}$ embryos does not occur in the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ embryos. It is conceivable that



pRb is capable of forming at least three distinct interchangeable complexes to regulate proliferation. Complete genetic disruption of the *Rb1* gene in *Rb1*$^{-/-}$ animals disrupts all three aspects of growth control by pRb. In *Rb1*$^{\Delta G/\Delta G}$ animals E2F activity is deregulated but ectopic S-phase entry is prevented by the ability of pRb to stabilize p27 through a direct interaction between Cdh1 and Skp2. *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ animals disrupt both these elements of proliferative control and as appear to have deregulated proliferative control and rapidly develop tumors. In contrast combination of E2F1 disruption with the ΔG mutation does not lead to drastic disruption of proliferative control or induce tumorigenesis.

Figure 4-9 Model of pRb proliferative control

the remaining proliferative control is mediated by the maintained regulation of E2F1 through the specific site. To address this possibility we are currently attempting to generate *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ *E2f1*$^{-/-}$ animals to characterize the effect of abrogating all three mechanisms of cell cycle control.

The importance of all the mechanisms of cell cycle control shown in figure 4.9 is supported by previous work implicating the utilization of three distinct binding sites on pRb to control proliferation. These sites were defined as the 'general site' to regulate E2F dependent transcription, the 'LXCXE site' that indirectly regulates p27 levels, and the 'specific site', which forms a unique interaction with E2F1. Disruption of the 'specific site' in this study by introducing the ΔG mutation into E2f1$^{-/-}$ background was not found to alter proliferative control or tumor suppression. It has been suggested that the 'specific site' functions predominately in the regulation of E2F1-induced apoptosis to regulate the transcription of pro-apoptotic targets of E2F1 (*8*). E2F1 is capable of maintaining an interaction with hyperphosphorylated pRb (ppRb) (*8*) and complexes between ppRb and E2F1 have been identified at pro-apoptotic promoters (*29*). Presumably, this implicates the E2F1 'specific site' in the regulation of a distinct set of E2F target genes. This is supported by the data from our current study, as E2F activity in fibroblasts is deregulated to a similar extent as *Rb1*$^{-/-}$ cells, suggesting that the pRb-E2F1 'specific' interaction has a limited contribution to the regulation of canonical cell cycle target genes. Future studies are investigating the ability of the 'specific' interaction to block apoptosis in the ΔG- mice and the effect this may have on tumorigenesis and sensitivity to DNA damage.

The noted defects in the lungs of *Rb1*$^{\Delta G/\Delta G}$ mice suggest that, in some contexts, deregulated E2F expression is sufficient to induce abnormal development. This fits with

the observation that neuroendocrine hyperplasia noted in chimeric *Rb1*$^{-/-}$ animals is suppressed by loss of E2F3 (*27*), suggesting that the correct differentiation of the lung epithelium is dependent upon appropriate E2F regulation. However, the abnormalities in lung structure do not worsen with age and the surviving *Rb1*$^{\Delta G/\Delta G}$ do not appear to have respiratory difficulties, and no lung tumors have been observed. Further, a defect in skeletal muscle is observed in both the *Rb1*$^{\Delta G/\Delta G}$ and the *Rb1*$^{-/-}$ mice that is characterized by reduced differentiation and cell death of the muscle fibers. This likely contributes to the embryonic lethality of the mice. Recent work has suggested that the defects in *Rb1*$^{-/-}$ myocytes cannot be rescued with ectopic expression of p27, suggesting that the defect is not due to an inability to appropriately exit the cell cycle, but rather the lack of *Rb1* results in mitochondrial dysfunction that induces cell death through apoptosis and autophagy (*30*). This suggests that while proliferative control is largely maintained in the *Rb1*$^{\Delta G/\Delta G}$ mice, the deregulated E2F activity may cause tissue-specific alterations for non-proliferative reasons.

Recent studies have suggested that, contrary to expectations, many cell types are capable of proliferating in the absence of the activator E2Fs 1-3 (*26, 31, 32*). However, proliferation in the absence of E2F1-3 results in the activation of p53 and results in apoptosis of the cells (*31, 32*). Previous to this work, E2F transcription factors were thought to be largely required to initiate the entry into S-phase and was supported by the embryonic lethality of E2F1-3 mice and proliferative defects in fibroblasts with conditional inactivation of E2F1-3 (*33, 34*). However, more recent studies have suggested that E2F1-3 have an essential role for sustaining proliferation but are dispensable for entry into the S-phase of the cell cycle (*26, 32*). Our current work further extends the

revision of the canonical model of E2F activity in that deregulation of E2F activity appears to be insufficient to induce ectopic proliferation. This is highlighted by proliferative control in the $Rb1^{\Delta G/\Delta G}$ mice and cells despite highly deregulated E2F target gene expression. Therefore, while E2F regulation has a critical role in maintaining proliferation of cells, pRb can stabilize p27 to maintain proliferative control in cells with deregulated E2F target gene expression.

P27 functions as a cyclin-dependent kinase inhibitor to block the activity of CDKs, which phosphorylate pRb and release bound E2Fs to drive S-phase entry. This would suggest that p27 functions largely upstream of pRb to regulate proliferation. Our current study suggests a role for p27 downstream of pRb that occurs independently of E2F regulation. This is supported by the acceleration of tumorigenesis in $Rb1^{+/-}$ $Cdkn1b^{-/-}$ animals (*21*), and the ability of ectopic expression of p27 to induce a partial arrest of $Rb1^{-/-}$ cells (*35*). The mechanism by which p27 regulates proliferation in the absence of pRb is likely through modulation of CDKs, as down regulation of CDK activity is also sufficient to arrest proliferation in the absence of pRb (*36*). The ability of p27 to regulate proliferation in the absence of E2F regulation suggests that E2F regulation must be combined with CDK activity to advance beyond the G_1 phase of the cell cycle.

Disruption of the human *RB1* gene results in the formation of highly penetrant retinoblastoma early in life and occurs frequently during non-small cell lung carcinoma development (*37*). The vast majority of patients with familial retinoblastoma carry genetic alterations of the human *RB1* gene that result in complete inactivation of the gene through large-scale genomic rearrangements, mutations that alter splicing or frameshift mutations to prevent the production of functional pRB protein (*38*). Relatively few

missense mutations of *RB1* are observed in human cancers and the few examples are located in regions that likely disrupt the stability of the entire protein (*39*). This is in stark contrast to the p53 tumor suppressor, which is typically inactivated in human cancer through a number of distinct missense mutations that localize to the DNA binding domain and other interfaces critical for its tumor suppressive properties (*40*). Our work suggests that single missense mutations in pRb would be insufficient to disrupt proliferative control as pRb is capable of engaging multiple mechanism to induce cell cycle arrest that are mediated by distinct structural interfaces. Thereby, this provides a molecular basis for the paucity of discrete loss of function missense *RB1* mutations in human cancer. Any point mutation of a surface exposed amino acid would likely be unable to disrupt all of the distinct binding sites that contribute to cell cycle control by pRb and tumor suppression.

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5 Discussion

5.1 Summary of findings

The retinoblastoma tumor suppressor protein was identified through close genetic analysis of patients with familial retinoblastoma. However, the specific molecular role for pRB in cancer and proliferative control is still unclear in many respects. Retention of functional pRB in many types of cancer is not well described by current models of pRB. Further, no mutations of pRB have been identified from human cancers that lead to discrete binding defects. In this thesis I utilize various experimental systems to dissect the functional roles of discrete binding sites in pRB to better understand their contribution to proliferative control and tumor supression.

Contrary to current understanding, I find that the ability of pRB to regulate proliferation extends beyond regulation of E2F transcriptional control. In chapter 4 we describe a novel gene-targeted mouse model, which provides evidence for the importance of p27 regulation by pRB and suggests that in the absence of E2F control, p27 can function to maintain proliferative control. As described in chapter 3, these pathways are controlled by distinct interaction surfaces on pRB, which suggests an explanation for the lack of E2F-binding deficient pRB mutants in human cancer. These findings suggest that complete pRB inactivation is required in human cancer to abrogate all mechanisms of cell cycle control that are controlled by distinct binding sites.

While cancers such as retinoblastoma and small cell lung cancer typically have complete genetic disruption of *RB1,* the vast majority of human cancers maintain the expression of functional pRB (*1*). These cancers bypass the ability of pRB to prevent ectopic S-phase entry through the deregulation of CDK complexes, which in turn maintain pRB in a hyperphosphorylated state. The selection for pRB maintenance in many human cancers is not well understood but may involve a protection from apoptosis (*2*). Current models of pRB function suggest that phosphorylated pRB is largely inactive however, we show in chapter 2 that pRB in its hyperphosphorylated state is able to maintain an interaction with E2F1 through the E2F1 'specific site'. Furthermore, the 'specific' interaction is capable of regulating the expression of the pro-apoptotic p73 promoter. This provides a potential mechanistic basis for the maintenance of pRB in human tumors as a means to restrain the apoptotic activity of E2F1. Taken together this thesis extends the current understanding of pRB function and provides experimental evidence that supports a multifaceted role for pRB in proliferative control and tumorigenesis.

5.2 An extended model for pRB function

This thesis provides experimental evidence for a refinement in the model of pRB in proliferative control and tumor suppression. As depicted in figure 5.1 this work highlights the ability of the discrete binding interfaces to mediate the overall functionality of pRB. Specifically, pRB is capable of forming a series of interchangeable complexes in distinct phases of the cell cycle. pRB can interact with E2F/DP through the 'general site' to block the transactivation domain of E2Fs (*3*). Further these complexes can form at E2F target

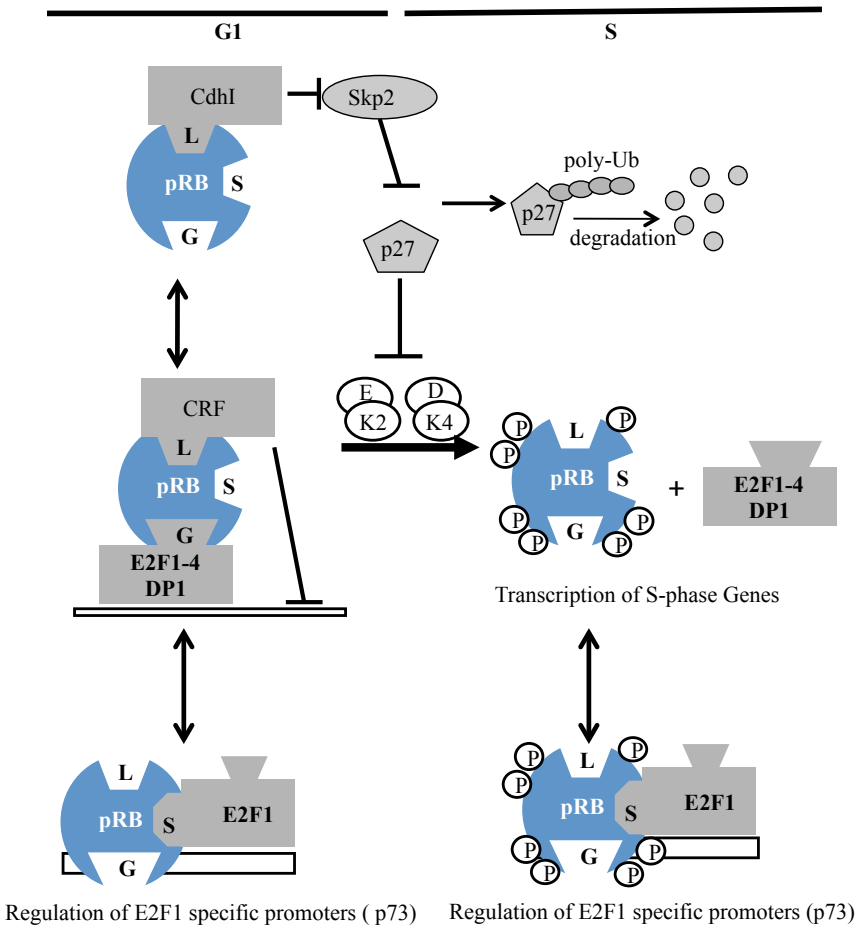

Figure 5-1 An extended model of pRB function

genes and recruit CRFs to actively repress E2F target gene expression(*4*). pRB can also form complexes with E2F1 through the specific site of pRB and the marked box domain of E2F1(*5*). As described in chapter 2 these two E2F binding sites are regulated in a distinct manner by phosphorylation. The 'general site' is disrupted by phosphorylation while the 'specific site' appears to be resistant to disruption by phosphorylation. Complexes between the 'specific site' and E2F1 retain the ability to regulate E2F1 specific targets that include p73 and may function to attenuate E2F1 induced apoptosis. These data provide a structural basis for the observed complexes between pRB and E2F1 in S-phase (*6*) and between phosphorylated pRB and E2F1 following DNA damage at pro-apoptotic promoters (*7*).

In addition to the interaction with E2Fs, pRB is capable of regulating p27 stability through an interaction with two ubiquitin ligase complexes that is dependent upon the LXCXE binding cleft (Fig. 5.1) (*8, 9*). As described in chapter 3 with the use of the ΔCRF mutant of pRB and in chapter 4 with the use of *Cdkn1b*$^{-/-}$ mice we find that the regulation of p27 functions in a redundant manner to control proliferation in the absence of E2F regulation. Disruption of both the regulation of E2Fs and p27 by pRB in the ΔG-ΔCRF-pRB reduces the arrest of Saos-2 cells (chapter 3) and the *Rb1*$^{\Delta G/\Delta G}$*Cdkn1b*$^{-/-}$ animals results in a loss of proliferative control and tumor formation in the mice (chapter 4). This suggests a model for pRB function at the G_1-S transition that more closely resembles a network rather than the conventional linear pathway (Fig. 1.4). Multiple binding sites appear to contribute to the overall function of pRB as a barrier to tumorigenesis.

5.3 Therapeutic potential of the 'specific site'

Given that the majority of human cancers express pRB that is phosphorylated by CDKs (*10*), our work suggests that ppRB-E2F1 complexes may exist in most human cancers. The 'specific site' appears to have a critical role in regulating E2F1-induced apoptosis (*5*) and complexes between phosphorylated pRB and E2F1 have been localized to apoptotic promoters in response to DNA damage (*7*). Our data in chapter 2 suggests that the complexes between phosphorylated pRB and E2F1 are likely mediated by the 'specific site' and thus implicates this site in the regulation of pro-apoptotic promoters following DNA damage. DNA damage-based therapies remain the mainstay of chemotherapy and function by rapidly killing proliferating cancer cells (*11*). The majority of human cancers have mutations that abrogate p53 (*12*) and as such the homologous p73 protein mediates apoptosis in cancer cells (*13*). In turn p73 is activated primarily by E2F1, as loss of E2F1 significantly impairs the p73 response to DNA damage (*14*). As the 'specific site' is able to regulate the activation of p73 by E2F1 and this interaction is likely maintained in most human cancers, this may provide a mechanism for cancer cells to attenuate the response to DNA damage.

A small molecule inhibitor designed to disrupt the interface between E2F1 and the 'specific site' could, in combination with conventional DNA damage-based chemotherapies, significantly increase the efficacy of cancer treatment. Given that the 'specific site' is independent of the 'general E2F site', the small molecule could be designed to only disrupt E2F1 when bound at the 'specific site'. Since the 'specific site' does not appear to have a major role in proliferative control (chapter 3), the drug would not likely interfere with proliferative control as the 'general site' could maintain

interaction with E2F1 to block ectopic proliferation. However, in cancer cells with pRB maintained in a hyperphosphorylated state, our work suggests that the 'general site' would be largely ineffective at regulating E2F1, which would be free to induce expression of p73 and other apoptotic targets in response to the DNA damage-based therapy.

A fluorescence resonance energy transfer (FRET) system could be used to screen molecular libraries for small molecule inhibitors of the 'specific site'. FRET paired proteins CFP and YFP have been cloned onto fragments of pRB and E2F to generate a robust assay to facilitate screening of these compounds. Once a molecule has been developed the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ mice would provide an ideal system to study the effectiveness of potential molecules in the treatment of cancer. These mice mimic the inactivation of pRB in the majority of human cancers as they disrupt E2F and p27 regulation but likely maintain the ability of ppRB to interact with E2F1. Therefore, the therapeutic utility of releasing E2F1 from the 'specific site' could be studied in the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ animals. Successful inhibitors of the 'specific site' would be expected to potentiate cell death after treatment with chemotherapy.

In addition to providing a potential novel means to target cancer cells, pRB may also provide a prognostic marker in human cancers. Contrary to expectations, some studies have reported that pRB expression is inversely correlated with survival in patients with ovarian cancer (*15*). Specifically, patients with elevated levels of pRB expression had a significantly worse prognosis compared with patients that lack expression of pRB. The patients were from a clinical study comparing the use of cisplatin and paclitaxel versus carboplatin and paclitaxel chemotherapy regimes, which are all designed to induce

DNA damage and cell death in malignant cells (*16*). It is possible that patients with maintained pRB expression block the activation of E2F1 to attenuate the effectiveness of DNA damage based therapy.

Another clinical scenario is in lung cancer where almost all cases of small cell lung cancer (SCLC) have complete disruption of pRB, while non-small cell lung cancers (NSCLC) maintain pRB expression (*10*). SCLC patients have a much better response to chemotherapy and tend to have a higher rate of apoptosis than NSCLC patients (*17*). These observations warrant further investigation into the role that maintained pRB expression might have on the regulation of E2F1 and apoptotic targets. Conceivably, the lack of pRB expression could provide a means to stratify patients that may respond best to chemotherapy. NSCLC tumors that do not express pRB respond more effectively to chemotherapy than the majority of cancers that maintain pRB expression (*18*). Therefore, while chemotherapy is not effective in the majority of NSCLC patients it is conceivable that chemotherapy would be an effective treatment for tumors that lack pRB expression (*19*). The majority of patients, which maintain pRB expression, would be potential candidates for therapies directed at the disruption of the 'specific site'. Since p53 mutations occur in up to 90% of NSCLCs the majority of apoptosis is likely induced by p73 (*13*). Given the strong induction of p73 by E2F1 (*14*) it suggests that therapies targeted to potentiate E2F1 activity may prove to be effective means to increase the chemosensitivity of this cancer.

5.4 Proliferative control in the absence of E2F and p27 regulation

As shown in chapter 4 the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ mice rapidly develop tumors with a similar incidence to the *Rb1*$^{+/-}$ *Cdkn1b*$^{-/-}$ animals, however in contrast to *Rb1*$^{-/-}$ animals, these mice are largely viable. The defect in *Rb1*$^{-/-}$ mice most closely associated with proliferative control is the placental defect in which ectopic proliferation of the placental trophoblast cells results in inadequate development and embryonic lethality of the mice (*20, 21*). Given that the *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ mice are born at the expected Mendelian ratios, this suggests that proliferative control in the placenta is largely maintained. However, the tumorigenesis and altered cell cycle kinetics observed in culture suggests that, in many contexts, proliferation is disrupted in these animals. As described in chapter 3 the 'specific site' can also contribute to proliferative control in the absence of the 'general site' and p27 regulation through the 'LXCXE' binding cleft. Therefore, the lack of placental defects may be due to the ability of the 'specific site' to maintain proliferative control and is currently being assessed through the generation of *Rb1*$^{\Delta G/\Delta G}$ *Cdkn1b*$^{-/-}$ *E2f1*$^{-/-}$ mice.

It is also possible that additional pathways exist outside of E2F1 regulation through the 'specific site' to maintain cell cycle control. In chapter 3 we utilized the ΔCRF mutant to disrupt the LXCXE binding cleft in pRB and the interaction with Cdh1 to prevent the stabilization of p27. However, in our gene-targeted mouse model we utilized the direct disruption of p27 to abrogate this pathway. It is possible that the LXCXE binding cleft may function through other means to ensure appropriate

proliferative control. pRB is thought to recruit chromatin remodeling enzymes through this binding site to E2F-target promoters to produce a closed chromatin landscape to actively repress the expression of these genes (*4*). Therefore, it is expected that in the absence of E2F binding chromatin remodeling factors will not be recruited to E2F target genes in $Rb1^{\Delta G/\Delta G}$ animals. This is supported by preliminary data that suggests that the $Rb1^{\Delta G/\Delta G}$ mice share similar defects to the $Rb1^{\Delta L/\Delta L}$ animals including a hyperplasia of the mammary ductal epithelium and a defective response to TGF-β(Data not shown). However, condensin and cohesion complexes have recently been shown to interact with pRB at the LXCXE binding cleft (*22, 23*). Cohesin and condensin complexes have been implicated in the direct transcriptional control of target genes involved in proliferation and differentiation (*24*). It is possible that pRB participates in these complexes with condensin and cohesin as a means to regulate proliferation in the $Rb1^{\Delta G/\Delta G}$ mice and warrants further investigation.

5.5 Perspectives

The work in this thesis has extended our understanding of the role of pRB in proliferative control and tumor suppression. Using a combination of *in vitro* interaction assays, cell culture experiments and mouse models, we have refined the model of pRB function. However, many aspects of pRB's function remain unclear. While both the 'general' and the 'specific site' appear to contribute to the regulation of E2F transcription factors, current data suggests that the 'specific site' may regulate a subset of E2F1 specific promoters. However, there is currently little experimental data that describes the ability of the 'general' or the 'specific site' to regulate distinct E2F targets. The ΔG

mouse model presented in chapter 4 allows for the disruption of only the 'general site' so that the 'specific site' can be studied in isolation. An analogous mouse model that disrupted the 'specific site' would allow for the 'general site' to be studied in isolation.

As outlined in chapter 2 multiple synthetic mutations were identified that disrupt the interaction between pRB and E2F1 at the 'specific site'. Further analysis suggests that the F839A substitution would be an ideal candidate to selectively disrupt the interaction between the 'specific site' and E2F1 (Data not shown). Development of a ΔS mouse model in which the 'specific site' was disrupted would greatly enhance our understanding of the molecular function of these sites. One potential application of these models is the use of chromatin immunoprecipitation sequencing (ChIP-Seq) to identify targets of the 'general' and 'specific sites'. The identification of targets would further our understanding into the molecular function of these sites. Furthermore, the identification of unique transcriptional targets for the 'specific site' would provide a potential role for the persistent ppRB-E2F1 complexes beyond the G_1 phase of the cell cycle.

The $Rb1^{\Delta G/\Delta G}$ mice will provide an ideal experimental system for future studies to investigate the molecular role of E2F regulation by pRB. Specifically the differentiation and development of specific tissues can be studied in greater detail. As described in chapter 4 the $Rb1^{\Delta G/\Delta G}$ mice have significant defects in muscle and lungs that is reminiscent of $Rb1^{-/-}$ animals. The lack of proliferative defects in the $Rb1^{\Delta G/\Delta G}$ mice suggests that the defects may occur independently of cell cycle defects. However, this warrants further investigation through the study of differentiation in $Rb1^{\Delta G/\Delta G}$ cells and embryos. The muscle development can be studied through the differentiation of MEFs into myoctyes through the expression of MyoD (*25*) Since $Rb1^{-/-}$ are defective in this

differentiation the *Rb1*$^{\Delta G/\Delta G}$ fibroblasts can be utilized to assess the role of E2F regulation in this process. In this way the ΔG mouse model will likely prove to be a valuable system to investigate the contribution of E2F regulation in multiple developmental contexts including muscle differentiation.

Previously, a gene-targeted mouse model has been created that introduces three substitutions into the mouse *Rb1* gene to selectively disrupt the interaction with proteins at the LXCXE binding cleft (*26*). These substitutions are termed ΔL and this mouse model has highlighted critical roles for the LXCXE binding cleft in response to TGF-β, senesence and in the maintenance of genome stability (*22, 27, 28*). The ΔG mouse model described in this thesis will further our understanding of the molecular basis for the role of pRB in these pathways. As depicted in figure 1.4, the current model for LXCXE function suggests that it is dependent upon the 'general site' to recruit pRB complexes to E2F target genes. However, studies that suggest a role for the LXCXE cleft in the regulation of pericentric heterochromatin and the maintenance of genomic stability (*22, 26*) raise the question of how pRB is targeted to regions that may lack E2F binding sites. By investigating the previously identified LXCXE dependent functions of pRB in the ΔG mouse model the requirement for E2F binding can be explored. Furthermore, if these pathways occur independently of E2F binding the models will afford the ability to potentially identify the alternative means by which pRB is targeted to distinct genomic loci.

Current understanding of pRB suggests that it functions as an adapter protein to nucleate distinct protein complexes to maintain proliferative control and mediate other cellular signaling. Since the binding sites of pRB mediate distinct complexes that can

produce contrasting signals there is a need to study the role of these binding sites in isolation. As such, this thesis has utilized multiple experimental models and developed a series of novel systems to better understand the functionality of the distinct binding interfaces of the retinoblastoma tumor suppressor protein.

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Appendix A: An overlapping kinase and phosphatase docking site regulates activity of the retinoblastoma protein

ARTICLES

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An overlapping kinase and phosphatase docking site regulates activity of the retinoblastoma protein

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The phosphorylation state and corresponding activity of the retinoblastoma tumor suppressor protein (Rb) are modulated by a balance of kinase and phosphatase activities. Here we characterize the association of Rb with the catalytic subunit of protein phosphatase 1 (PP1c). A crystal structure identifies an enzyme docking site in the Rb C-terminal domain that is required for efficient PP1c activity toward Rb. The phosphatase docking site overlaps with the known docking site for cyclin-dependent kinase (Cdk), and PP1 competition with Cdk-cyclins for Rb binding is sufficient to retain Rb activity and block cell-cycle advancement. These results provide the first detailed molecular insights into Rb activation and establish a novel mechanism for Rb regulation in which kinase and phosphatase compete for substrate docking.

The retinoblastoma tumor suppressor protein (Rb) regulates the cell cycle through its capacity to associate with and influence the function of a number of cellular proteins. In the best-characterized example, Rb binds and inhibits E2F transcription factors to coordinate the initiation of S phase with mitogenic signaling[1,2]. Rb activity—namely, its competency to bind E2F—is regulated by phosphorylation in a cell cycle–dependent manner. In G0 and early G1, Rb is active and modified at relatively few phosphorylation sites. In this hypophosphorylated state, Rb sequesters E2F and recruits transcriptional corepressors and chromatin-modifying enzymes to E2F-responsive promoters to block transcription[1,3–6]. Hyperphosphorylation of Rb by cyclin-dependent kinases (Cdks) from late G1 until mitosis inactivates Rb by dissociating these factors and results in the expression of genes required for DNA synthesis and cell-cycle progression[1,7,8]. The enzyme protein phosphatase 1 (PP1), which is required for mitotic exit and is responsible for reversing the phosphorylation of many Cdk substrates, dephosphorylates Rb beginning in anaphase[9–11]. PP1-dependent Rb dephosphorylation has also been observed during S and G2 in response to hypoxia and DNA damage, suggesting that it is also responsible for Rb activation under these conditions[12,13]. The importance of phosphorylation for regulating Rb activity as a tumor suppressor is underscored by the fact that genes encoding cyclin D and p16 are frequently mutated in cancers, leading to constitutive Rb hyperphosphorylation[14,15].

Mechanisms for regulating Rb phosphorylation have focused on the modulation of Cdk activity[1,2,16]. Although levels of Rb phosphorylation in the cell cycle generally coincide with levels of Cdk activity, there are circumstances, such as during mitotic exit and after DNA damage, in which Rb must be actively dephosphorylated and maintained in a hypophosphorylated state. In fact, several cancer lines have been shown to be defective in activating Rb by dephosphorylation[17]. Therefore, an important mechanistic question remains regarding how phosphatase activity opposes kinase activity to control Rb phosphorylation. A stable PP1–Rb complex has been observed that is coincident with the timing of dephosphorylation in mitosis[18]. Nevertheless, compared to Cdks, much less is known regarding how PP1 recognizes Rb and how Rb dephosphorylation may be regulated.

In cells, PP1 activity typically arises from a complex containing the catalytic subunit (PP1c) and a variable regulatory subunit; the latter confers substrate specificity and enhances activity[19]. There are three mammalian isoforms of PP1c; the isoforms all contain the highly conserved catalytic domain and only differ in their unstructured N and C termini[19,20]. Nearly all regulatory subunits and many inhibitors contain a consensus Arg-Val-x-Phe (RVxF) sequence, which binds PP1c at a site distinct from the catalytic site[19–21]. Endogenous Rb–PP1 complexes copurify with other proteins, and an interaction between Rb and the myosin phosphatase-targeting subunit has been reported[22,23]; these observations suggest the existence of a regulatory subunit for Rb dephosphorylation. In contrast, there have been several reports of a direct, functional complex between Rb and all three PP1c isoforms without the requirement of a targeting subunit[24,25]. Thus, the mechanism of Rb-specific PP1 activity remains unclear.

Rb contains two structured domains known as the N-terminal and pocket domains as well as a C-terminal domain (RbC) of ~150 residues (**Fig. 1a**). RbC is necessary and sufficient for observation of an Rb–PP1c complex in cell extracts for all three PP1c isoforms[24,25]. RbC is intrinsically disordered but adopts structure upon binding E2F-DP heterodimers[26] (**Supplementary Fig. 1**). Other proteins that have been shown to associate with RbC include cyclins, Skp2, c-Abl and MDM2 (refs. 27–30). In the case of cyclin A (CycA), a crystal structure

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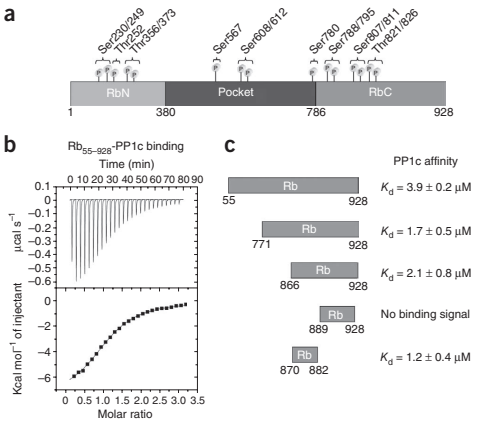

Figure 1 $Rb_{880–892}$ is necessary and sufficient for PP1c association. (**a**) Domain structure of Rb with the location of the conserved Cdk consensus phosphorylation sites. (**b**) Isothermal titration calorimetry (ITC) data for titration of $Rb_{55–928}$ into PP1c. (**c**) Results from ITC experiments as shown in **b** but with RbC truncation mutants. Sample ITC data from each experiment are shown in **Supplementary Figure 2**.

reveals that a short $Rb_{868–878}$ peptide docks to the structured CycA domain in an extended conformation[31]. This sequence contains the canonical Arg-x-Leu (RxL) sequence motif that targets Cdk-cyclins to Rb and other substrates for efficient phosphorylation[27,32]. At present, little is known about whether these RbC binding partners are capable of interacting with Rb simultaneously or competitively, leaving their regulatory impact on Rb uncharacterized.

We examine here the RbC-PP1c association in molecular detail to understand the mechanism of Rb activation by dephosphorylation. We find that human PP1c uses its regulatory subunit-binding cleft to dock with an RVxF-like motif in RbC. The PP1c binding sequence overlaps with the previously identified RxL cyclin binding site, and the association of Rb with PP1c and Cdk-cyclin is exclusive. These results reveal an efficient regulatory mechanism, generally applicable in cell signaling, in which phosphatase and kinase activities affect phosphorylation state not only through catalysis but also through restricting access to their target substrate.

RESULTS

$Rb_{870–882}$ is necessary and sufficient for PP1c association

To determine the precise sequence requirements for RbC-PP1c binding, we applied isothermal titration calorimetry (ITC) to quantitate binding affinity. We titrated recombinant, purified Rb proteins into recombinant PP1c (α-isoform), and we calculated dissociation constants from the resulting isotherms (**Fig. 1**). We first determined that $Rb_{55–928}$, which contains all of the conserved Rb domains and phosphoacceptor sites, binds PP1c with $K_d = 3.9 \pm 0.2$ µM (**Fig. 1b,c**). This value is typical for enzyme-substrate binding interactions and is similar to that previously observed between an RbC peptide and CycA[31].

We next made a series of truncation mutants and tested the affinity of these mutants for PP1c by calorimetry (**Fig. 1c** and **Supplementary Fig. 2**). $Rb_{771–928}$ and $Rb_{866–928}$ bind PP1c with similar affinity to that of full-length Rb, which is consistent with previous reports that RbC is sufficient for the association and phosphorylation is not required[24,25,33]. Titration of $Rb_{889–928}$ into PP1c results in no detectable heat signal, indicating that the conserved residue sequence between 866 and 889 is required for binding. Using a synthetic peptide, we found that $Rb_{870–882}$ binds PP1c with comparable affinity (K_d = 1.2 ± 0.4 µM) to that of full-length Rb, confirming that $Rb_{870–882}$ is necessary and sufficient for PP1c association and likely contains all of the significant interacting residues. This conserved sequence contains the CycA docking site and a Lys-Leu-Arg-Phe (KLRF) sequence that resembles the consensus RVxF motif found in PP1 regulatory subunits (**Supplementary Fig. 1**).

Crystal structure of $Rb_{870–882}$-PP1c

We next crystallized and solved the structure of a complex of the α-isoform of PP1c with an $Rb_{870–882}$ peptide (**Table 1**, **Supplementary Methods** and **Supplementary Fig. 3**). The structure of PP1c in the complex is essentially identical to that observed in both the PP1c–microcystin and PP1c–tungstate complexes[33,34]. The Rb peptide binds PP1c in an extended conformation at the hydrophobic interface of the core β-sandwich subdomain opposite the catalytic site (**Fig. 2a**). Rb binding is mediated both by main chain hydrogen bonding and

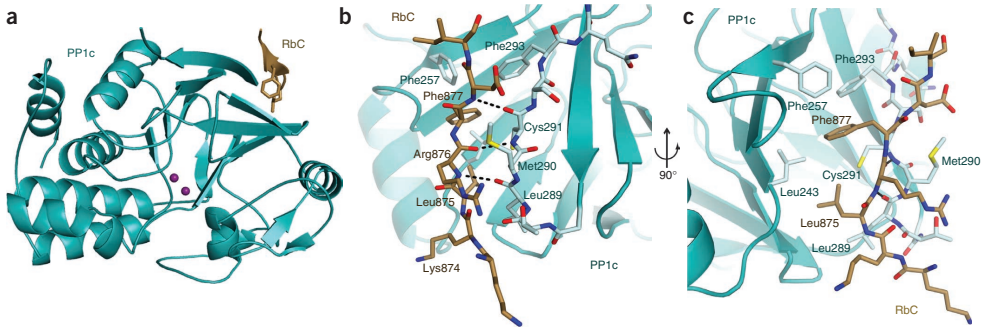

Figure 2 Structure of the $Rb_{870–882}$–PP1c complex. (**a**) RbC (brown) binds in an extended conformation and extends sheet 1 of the PP1c β-sandwich domain (cyan). Purple spheres, Mn^{2+} ions at the distant PP1c catalytic site. (**b**) Close-up view of the $Rb_{870–882}$–PP1c interface. The main chain hydrogen bonding interactions between the RbC peptide (light brown) and PP1c (cyan) are shown. (**c**) Hydrophobic side chain interactions between $Rb_{870–882}$ and PP1c.

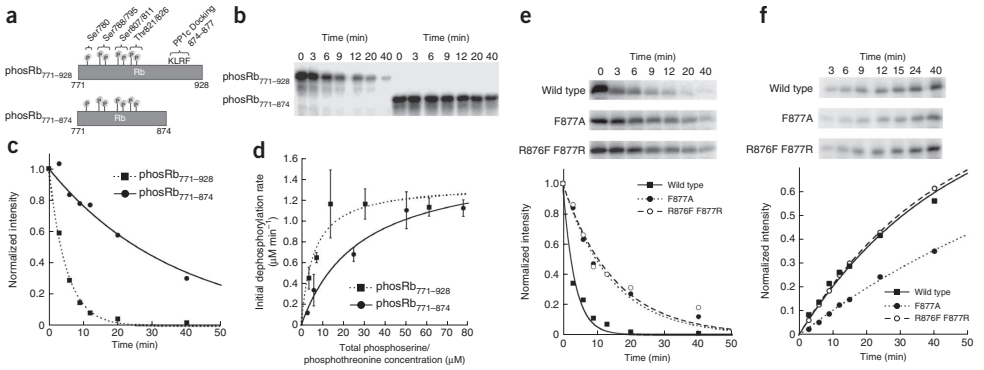

Figure 3 The RbC KLRF docking sequence is required for efficient dephosphorylation by PP1c. (**a**) RbC constructs used as substrates in the phosphatase assays. (**b**) PP1c phosphatase assay using 5 μM ^{32}P-labeled phosRb$_{771–928}$ and phosRb$_{771–874}$ and 10 nM PP1c. Quenched aliquots removed from the reaction at the indicated time point are visualized with phosphorimaging. (**c**) Plot of band intensities in **b** as a function of reaction time. (**d**) Plot of initial reaction rate as a function of substrate concentration for dephosphorylation of phosRb$_{771–928}$ and phosRb$_{771–874}$. Fit of the data to a simple steady-state model indicates similar apparent k_{cat} values; however, the apparent K_M for phosRb$_{771–874}$ is greater. Error bars, fitting error for initial rate calculation from reaction time course data. (**e**) Analysis of docking-site mutations in the phosphatase assay described in **b** and **c**. phosRb$_{771–928}$ with the indicated mutation was used as a substrate at a concentration of 1 μM with 10 nM PP1c. (**f**) Analysis of docking-site mutations in a Cdk kinase assay; 2 μM of the indicated Rb$_{771–928}$ substrate was phosphorylated with Cdk2-CycA in the presence of E2F1-DP1.

hydrophobic side chain interactions (**Fig. 2b**,**c**). Arg876–Asp878 of Rb form a short β-strand that adds to sheet 1 of the PP1c β-sandwich subdomain. The Rb β-strand makes hydrogen bonding interactions with the edge strand of the sheet that are typical of parallel strand-strand interactions.

The other significant interactions between the Rb peptide and PP1c are made by the highly conserved hydrophobic side chains of Leu875 and Phe877 (**Fig. 2c** and **Supplementary Fig. 1**). Each inserts into a pocket within the hydrophobic core of the β-sandwich subdomain of PP1c. The PP1c β-sandwich structure and the specific side chains that contact RbC are conserved in all three mammalian isoforms of the enzyme (**Supplementary Fig. 4**). Thus, our structural data are consistent with and explain the previous observation that all of the PP1c isoforms bind Rb[25]. Furthermore, the observation that RbC contacts PP1c at a site that is distinct from the phosphatase active site explains the observation that catalytic activity of PP1c is not required for Rb-PP1 association[35].

The location of the Rb peptide binding site in PP1c and the molecular interactions stabilizing the complex are nearly identical to those observed between PP1c and the RVxF motif of two PP1 targeting subunits[20,21]. In the structure of the myosin phosphatase subunit 1 (MYPT1) bound to PP1c (isoform δ) (**Supplementary Fig. 5**), Lys37–Asp39 of MYPT1 add to the PP1c sandwich domain as a parallel β-strand, and Val36 and Phe38 of MYPT1 insert into the same hydrophobic pockets of PP1c as observed here for Leu875 and Phe877 of Rb[20]. Notably, the occurrence of leucine in RVxF motifs is extremely rare, and mutation of the canonical valine to leucine sometimes abolishes docking motif binding[36]. However, the similarity of contacts by RbC and MYPT1 with PP1 shows that the KLRF sequence at residues 874–877 of Rb functions as an RVxF motif.

It is noteworthy that Leu875 and Phe877 in RbC also bind to hydrophobic pockets in CycA (**Supplementary Figs. 1** and **5**)[31]. Leu875 is the leucine in the Rb RxL motif that is required for its phosphorylation[27,31,32]. Phe877 is buried along with Leu875 in the RbC-Cdk2-CycA structure, and both appear critical for stabilizing the observed docking interaction between kinase and substrate[31]. We found that mutation of these hydrophobic residues results in a loss of RbC affinity for PP1c and Cdk2-CycA (**Supplementary Methods** and **Supplementary Fig. 2**). These experiments verify that Leu875 and Phe877 are part of an enzyme-docking site in RbC required for association with both enzymes.

The Rb paralogs p107 and p130 also contain RxL sequences that are critical for binding to Cdk2-CycA[31,32]. However, unlike Rb, the phenylalanine in both the p107 and p130 docking motifs directly follows the leucine (Arg-Arg-Leu-Phe (RRLF)). We found that the CycA binding motifs in both pocket proteins (p107$_{655–667}$ and p130$_{677–689}$) do not also bind PP1c (**Supplementary Fig. 2**). This result is consistent with the crystal structure, which reveals that leucine forms critical contacts with PP1c in the −2 position (relative to the phenylalanine).

PP1c docking is required for efficient RbC dephosphorylation

To examine the effects of the Rb-PP1c association on Rb-directed PP1 phosphatase activity, we developed an assay to measure Rb dephosphorylation rates. We quantitatively phosphorylated two RbC constructs, both containing seven Cdk consensus sites (**Fig. 3a**), with ^{32}P. After mixing substrate with phosphatase, signal intensity remained at longer time points in phosphorylated Rb$_{771–874}$ (phosRb$_{771–874}$) compared to phosRb$_{771–928}$ (**Fig. 3b**), indicating that deletion of the PP1c docking site in RbC results in a loss of dephosphorylation efficiency. Quantification of the signal indicates that the first-order rate constant for dephosphorylation of phosRb$_{771–874}$ ($k_{dephos} = 0.027 \pm 0.002$ min^{-1}) is approximately eight times smaller than for phosRb$_{771–928}$ ($k_{dephos} = 0.20 \pm 0.01$ min^{-1}) (**Fig. 3c**). We also found that a short peptide containing the KLRF sequence inhibits phosRb$_{771–928}$ dephosphorylation when added to the assay, further confirming that the docking site permits more efficient substrate processing (**Supplementary Fig. 6**). Analogous phosphatase assays with mutant phosRbC fragments

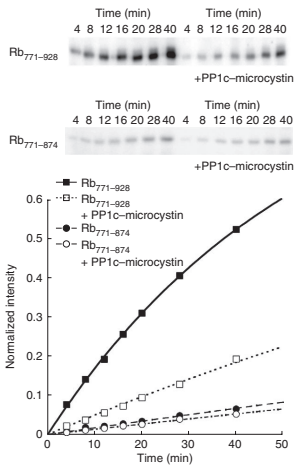

Figure 4 PP1c inhibits Cdk2-CycA activity toward RbC. Phosphorylation of 2 μM $Rb_{771–928}$ or $Rb_{771–874}$ with 75 nM Cdk2-CycA in the absence and presence of a saturating concentration of PP1c–microcystin (15 μM).

that contain only one pair of phosphorylated sites show that dephosphorylation occurs at all of the sites with kinetics that are sensitive to the presence of the PP1c docking site (**Supplementary Fig. 7**).

We performed phosphatase assays at different substrate concentrations to determine apparent steady-state kinetic parameters for dephosphorylation of the multiple RbC sites (**Fig. 3d**). The apparent k_{cat} for dephosphorylation of phos$Rb_{771–874}$ (k_{cat} = 140 ± 20 min^{-1}) and of phos$Rb_{771–928}$ (k_{cat} = 160 ± 20 min^{-1}) are similar. However, the apparent K_M for phos$Rb_{771–874}$ (K_M = 30 ± 10 μM) is greater than the apparent K_M of phos$Rb_{771–928}$ (K_M = 6 ± 3 μM). These results are consistent with the RbC KLRF docking sequence enhancing dephosphorylation by enabling PP1c to capture substrate and form an enzyme–substrate complex.

We next examined how mutations in the overlapping PP1c and Cdk-cyclin docking site affect enzyme activity toward Rb. In the phosphatase assay, dephosphorylation of $Rb_{771–928}$ that contains an F877A mutation (k_{dephos} = 0.071 ± 0.004 min^{-1}) is slower than wild type (k_{dephos} = 0.29 ± 0.03 min^{-1}) (**Fig. 3e**). Switching the position of the phenylalanine and arginine (R876F F877R) in $Rb_{771–928}$, which creates a docking sequence that more resembles P107 and P130, also results in a smaller first-order rate constant in the assay (k_{dephos} = 0.067 ± 0.006 min^{-1}).

To test these mutants in a kinase assay, we prepared complexes of RbC and E2F1-DP1 to mimic the physiological, active Rb substrate. In binding assays, the presence of E2F1-DP1 does not affect appreciably the affinity of either PP1c or Cdk2-CycA for RbC (**Supplementary Fig. 2**), indicating that the E2F-DP binding site in RbC does not overlap with the common enzyme-docking site. The first-order rate constant characterizing phosphorylation of wild-type RbC (k_{phos} = 0.023 ± 0.001 min^{-1}) is greater than that for the F877A mutant (k_{phos} = 0.011 ± 0.001 min^{-1}) (**Fig. 3f**). This measured kinetic difference is consistent with the Lys-x-Leu-x-Phe (KxLxF) motif at 873–877 in Rb being required for phosphorylation by Cdk2-CycA[27]. The R876F F877R mutant (k_{phos} = 0.024 ± 0.001 min^{-1}) has a first-order rate constant similar to that of wild type. This observation follows previous findings that Cdk2-CycA is capable of docking to both K/RxLxF (Rb-like) or K/RxLF (p107-like) sequences[31]. Our kinetic studies of docking-site mutants show that both PP1c and Cdk-cyclin utilize an overlapping docking site in Rb and indicate that the R876F F877R mutant is defective as a PP1 substrate but not as a Cdk substrate.

PP1 inhibits Cdk2-CycA activity toward Rb

Considering that both kinase and phosphatase cannot bind the required docking site together, we hypothesized that each enzyme would act as an inhibitor of the other by occluding the site. We first tested whether inactive PP1c could inhibit the phosphorylation of RbC by Cdk2-CycA in the kinase assay (**Fig. 4**). In the absence of PP1c, the first-order rate constant for $Rb_{771–928}$ phosphorylation (k_{phos} = 0.0185 ± 0.0001 min^{-1}) was 11 times greater than that for $Rb_{771–874}$ phosphorylation (k_{phos} = 0.0017 ± 0.0001 min^{-1}). We then carried out kinase reactions in the presence of saturating quantities of PP1c that was irreversibly inhibited at its catalytic site with microcystin (**Fig. 4**). The presence of PP1c–microcystin reduces the rate constant for $Rb_{771–928}$ phosphorylation (k_{phos} = 0.0051 ± 0.0001 min^{-1}) such that it is more similar to the rate constant for $Rb_{771–874}$ phosphorylation. By contrast, PP1c–microcystin has little effect on $Rb_{771–874}$ phosphorylation (k_{phos} = 0.0013 ± 0.0001 min^{-1}). Thus, our data indicate that PP1c directly inhibits RbC phosphorylation by Cdk2-CycA and that inhibition is independent of phosphatase activity and dependent on the presence of the KLRF docking site. We have also found, using the phosphatase assay, that Cdk2-CycA inhibits RbC-directed PP1c phosphatase activity (**Supplementary Fig. 8**).

Inhibition of Cdk access to Rb blocks cell-cycle progression

Having established that Cdk and PP1c compete for Rb access, we investigated the functional importance of this competition in the context of cell-cycle regulation. The human osteosarcoma cell line Saos-2 is deficient for Rb, and Rb re-expression leads to a strong G1 arrest[37]. Coexpression of Cdk2-CycA abrogates this arrest through phosphorylation and inactivation of Rb[38,39]. We used this model system to observe the effect of PP1 on Cdk regulation of Rb (**Fig. 5a**). We found that the Rb-induced arrest was overcome by Cdk2-CycA expression, and it could be largely recovered by expressing PP1c. Notably, coexpression of a catalytically inactive mutant of PP1c (PP1c H248K) also resulted in a restoration of G1 arrest. Omission of Rb from these assays abrogated the PP1c-dependent cell-cycle block, confirming that Rb is the relevant target of enzyme competition. In **Figure 5b**, the expression levels of PP1c were titrated and reveal that catalytically inactive enzyme is as potent as wild type in blocking cell-cycle advancement under conditions in which Rb expression has been reduced. Based on these cell-cycle control data, we conclude that the competition for substrate access between Cdk2-CycA and PP1c on Rb offers an efficient means to control cell proliferation beyond the catalytic regulation of phosphorylation.

We next confirmed that PP1c inhibits phosphorylation of Rb in cells, as in our kinetic analyses, in a manner that is independent of catalytic activity. We used C33A cells to test whether exogenously introduced PP1c could compete with Cdks and block Rb phosphorylation regardless of cell-cycle position effects on enzyme activity (**Fig. 5c**). Ectopically expressed Rb becomes phosphorylated in C33A cells. Expression of a dominant negative Cdk2 controls for inhibition of Rb phosphorylation in our analysis, and coexpression of Rb with Cdk2-CycA shows the maximum extent of Rb hyperphosphorylation.

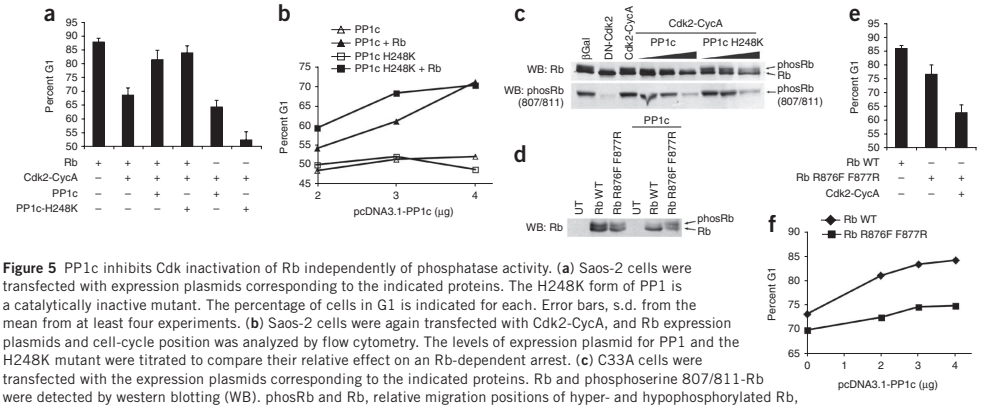

Figure 5 PP1c inhibits Cdk inactivation of Rb independently of phosphatase activity. (**a**) Saos-2 cells were transfected with expression plasmids corresponding to the indicated proteins. The H248K form of PP1 is a catalytically inactive mutant. The percentage of cells in G1 is indicated for each. Error bars, s.d. from the mean from at least four experiments. (**b**) Saos-2 cells were again transfected with Cdk2-CycA, and Rb expression plasmids and cell-cycle position was analyzed by flow cytometry. The levels of expression plasmid for PP1 and the H248K mutant were titrated to compare their relative effect on an Rb-dependent arrest. (**c**) C33A cells were transfected with the expression plasmids corresponding to the indicated proteins. Rb and phosphoserine 807/811-Rb were detected by western blotting (WB). phosRb and Rb, relative migration positions of hyper- and hypophosphorylated Rb, respectively. (**d**) C33A cells were transfected with the indicated proteins, and Rb was detected as in **c**. WT, wild type. (**e**) Saos-2 cells were transfected with expression plasmids corresponding to the indicated proteins, and the analysis was conducted as in **a**. (**f**) Saos-2 cells were transfected with Rb, or the indicated Rb mutant, and Cdk2-CycA expression plasmids as in **b**. Increasing quantities of PP1c were co-transfected to assess the sensitivity of the Rb mutant to protection from phosphorylation and subsequent cell-cycle advancement out of the G1 phase.

As predicted, expression of PP1c or catalytically dead PP1c H248K inhibited Rb phosphorylation levels in a dose-dependent manner.

Our kinetic data indicate that the Rb R876F F877R mutant is a poor PP1 substrate but a good Cdk substrate. We used this mutation to study the importance of the docking site for dephosphorylation and Rb activation in cell-based assays. First, we transfected wild-type Rb and Rb R876F F877R into C33A cells with and without also transfecting PP1c (**Fig. 5d**). Coexpression of PP1c reduces the observed phosphorylation of wild-type Rb (migrates as a faster, single band), whereas the mutant Rb R876F F877R is unaffected by phosphatase expression. This observation suggests that the docking interaction observed in our crystal structure is required in cells for efficient Rb dephosphorylation by PP1.

We also tested the Rb R876F F877R mutant in the Saos-2 cell-cycle arrest assay. Expression of Rb R876F F877R in Saos-2 cells gives a less robust arrest in G1 compared to wild type, consistent with the idea that Rb activation requires docking-dependent PP1c dephosphorylation that is defective in this mutant (**Fig. 5e**). Cdk2-CycA expression still inactivates Rb R876F F877R as expected because the kinase-docking site remains intact. We also find that, under conditions in which Rb and kinase are expressed, coexpression of PP1c is sufficient to restore the activity of wild-type Rb but is unable to reactivate phosphorylated Rb R876F F877R (**Fig. 5f**). Taken together, these data highlight a critical role for the KLRF docking site in the regulation of Rb activity.

Stable Rb–PP1 complexes are coincident with Rb activation

The competition for access to Rb between PP1c and Cdk-cyclin suggests that Rb and PP1c are in a complex at times when Rb is activated by dephosphorylation. To investigate the relevance of this mechanism of cell-cycle regulation under endogenous conditions, we examined the abundance of Rb–PP1c complexes in CV-1 cells during mitotic exit and transfected Saos-2 cells that arrest in a PP1-dependent manner in early G1 (**Fig. 6**). We used CV-1 cells because synchronization experiments have shown that PP1 and Rb associate selectively in late mitosis, coincident with Rb dephosphorylation and activation in these cells[18]. We first compared the relative level of the endogenous proteins in CV-1 cells with the level of transfected proteins in Saos-2 cells by applying recombinant standards (**Fig. 6a**). We found that the molar quantities of Rb in extracts from CV-1 and Saos-2 transfected cells were equivalent. Considering that the majority of Saos-2 cells are transfected in our experiments, the Rb expression level in the Saos-2 cells is no more than 2 times higher. The levels of PP1c in mitotic CV-1 cells were a little less than half as much as those of Rb, whereas the total levels of endogenous and exogenously introduced PP1c in arrested Saos-2 cells were approximately equivalent to those of Rb. These observations indicate that our transfection-based assay system closely mimics the levels of endogenous proteins under conditions where Rb is activated.

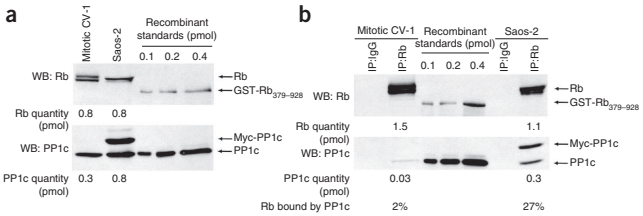

Figure 6 Abundant Rb–PP1c complexes during PP1c-dependent growth arrest. (**a**) Saos-2 cells were transfected as in **Figure 5a** to generate a PP1c-dependent arrest in early G1. CV-1 cells were released form an S-phase block, and mitotic cells were isolated by a mitotic shakeoff 16 h later. Extracts were analyzed by SDS-PAGE and western blotting (WB) to quantitate Rb and PP1c levels. Quantities of Rb and PP1c were determined by band intensities relative to a standard curve generated using recombinant proteins. The quantities are listed below each respective gel lane. (**b**) Rb was immunoprecipitated from extracts prepared as in **a**, and the quantities of Rb and associated PP1c were determined as above.

Table 1 X-ray data collection and structure model refinement statistics

	$Rb_{870-882}$-PP1c
Data Collection	
Space group	$P4_12_12$
Cell dimensions	
a, *b*, *c* (Å)	92.95, 92.95, 192.38
Resolution (Å)	83.6–3.2
R_{pim} (%)[a]	5.6 (20.6)
$I / \sigma I$	18.8 (4.6)
Completeness (%)	98.2 (97.8)
Redundancy	12.3
Refinement	
Resolution (Å)	3.2
No. Reflections	13,588
R_{work} / R_{free} (%)	22.1 / 26.1
No. Atoms	4,798
Protein	4,792
Ligand/ion	6
Avg. *B*-factor (Å^2)	50.1
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.789

Values in parentheses correspond to the highest-resolution shell (3.4–3.2 Å).
[a]$R_{pim} = \Sigma_{hkl} [1/(N-1)]^{1/2} \Sigma_i |I_i(hkl) - I(hkl)| / \Sigma_{hkl} \Sigma_i I_i(hkl)$, where *i* indexes the *i*th measurement of reflection hkl and *N* indicates the total number of times a given reflection is measured.

We next immunoprecipitated Rb complexes from CV-1 and transfected Saos-2 cells and immunoblotted for bound PP1c (**Fig. 6b**). The amount of PP1 coprecipitated from arrested Saos-2 cells is ~30% of the total amount of immunoprecipitated Rb, indicating that one-third of Rb molecules are bound to PP1c when cells are arrested in a PP1-dependent manner. The amount of PP1c coprecipitated with Rb in CV-1 cells is ~2%; however, given that the population of CV-1 cells is actively progressing through mitosis and the limitations of synchronization by shakeoff, this measurement likely underestimates the quantity of Rb–PP1c complex that exists in a cell at the instant of Rb activation. Taken together, these experiments suggest that PP1 can form stable, abundant complexes with Rb at endogenous expression levels. These complexes attenuate the activity of Cdks by blocking their access to Rb and regulate progression through the cell cycle.

DISCUSSION

Although much attention has been paid to the inactivation of Rb by Cdk phosphorylation from G1 through mitosis, less is known regarding how Rb is activated by PP1 dephosphorylation during mitotic exit and following cellular stress. We have identified a short sequence in RbC that binds to PP1c directly and is required for efficient Rb-directed PP1 phosphatase activity. Our structural data show that the molecular interactions stabilizing RbC–PP1c are nearly identical to those observed between PP1c and its regulatory subunits. Whereas PP1c typically uses its hydrophobic binding cleft to recruit an additional subunit responsible for substrate binding, here PP1c uses the cleft to recruit Rb substrate directly[20,21]. Although uncommon, a direct interaction between PP1c and the PP1 substrate Cdc25 has also been observed in *Xenopus laevis* embryonic extracts[40]. Recent data indicate that PP1c dissociates from inhibitors following Cdk inactivation during mitosis[9]. The timing of this population of free PP1c is concurrent with the requirement to dephosphorylate Rb through a direct interaction, and it would be interesting to explore whether other PP1 substrates are dephosphorylated at mitotic exit without a targeting subunit.

The fact that the direct enzyme-substrate association is mediated through the 'RVxF'-binding cleft may explain why an Rb-targeting regulatory subunit has not been identified and is not necessary for Rb dephosphorylation[25]. Although the existence of such a subunit cannot be ruled out, our data indicate that both Rb and a hypothetical Rb-targeting subunit could not both occupy the RVxF-binding cleft and that a different mode of Rb–PP1 holoenzyme assembly would be required. However, considering that multiple phosphates in varying sequence contexts must be hydrolyzed in Rb, it seems reasonable that the Rb phosphatase would not use a targeting subunit to impart a high degree of specificity. RbC closely mimics regulatory subunits in PP1 binding, and it is also tempting to speculate that Rb itself is a PP1 regulatory subunit, either sequestering nuclear PP1c from other activating subunits or regulating PP1c access to other substrates. Notably, it has been reported that RbC can act as a noncompetitive inhibitor of PP1c activity toward a generic substrate[35].

Our data, together with previous results, indicate that PP1c and Cdk2-CycA bind an overlapping docking site in RbC that is required in each case for efficient enzymatic activity[27,31]. To our knowledge, this observation is the first example of a PP1c-binding sequence (RVxF or other) having an additional functional role that competes with PP1 activity. As a result of their exclusive associations with substrate, we have shown that Cdk2-CycA and PP1c can each directly inhibit the activity of the other enzyme toward Rb. This result reveals a novel mechanism for the regulation of Rb phosphorylation state in which kinase and phosphatase compete for access to substrate. Given the conservation of the RxL binding cleft in cyclin paralogs, it is assumed that the observed competition would exist between PP1 and all Cdk-cyclins that phosphorylate Rb. In contrast, the other Rb family proteins p107 and p130 do not bind PP1c; this competitive mechanism is unique to Rb.

Competition between kinase and phosphatase for controlling the phosphorylation state of a common substrate has been established as an important mechanism in cell signaling, and a theoretical framework has been crafted for how such competition can generate critical signaling properties such as sensitivity, switch-like responses and multiple steady-state outputs[41–44]. However, few experimental observations of these properties have been reported. Our finding of a Cdk-cyclin competition with PP1c for Rb as a substrate not only provides a rare example of direct kinase-phosphatase competition but also shows that competition can be for substrate docking as well as catalysis. Notably, the presence of common kinase/phosphatase docking sites in mitogen-activated protein kinases has been observed[45], suggesting that competition for substrate binding may have a more general role in signal transduction.

In the context of Rb phosphorylation in cell-cycle control, signaling sensitivity and specificity are critical. From mitosis through G1, the capacity of PP1c to inhibit Cdk-cyclin could facilitate efficient Rb dephosphorylation in response to small changes in PP1c concentration and could prevent Rb from being promiscuously rephosphorylated by residual Cdk activity. The same holds true in response to cellular stress and cell-cycle exit, and in fact, it has been shown that Rb is dephosphorylated in response to DNA damage despite the presence of active Cdks[13]. These regulatory concepts that would serve to activate Rb are supported by our cell-cycle arrest assays. Therefore, our findings establish a biochemical mechanism through which Rb phosphorylation and function can be tightly controlled in the cell by directly competing kinase and phosphatase activities. Further study is necessary to determine what mechanisms influence the outcome of

the competition and how access of each enzyme to the docking site is controlled. Considering the observation that the association between Rb and PP1c is direct, the nuclear concentration of PP1c, free of inhibitors and other targeting subunits, is an intriguing possible factor.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Accession codes. Protein Data Bank: Coordinates and structure factors for the $Rb_{870–882}$-PP1c complex have been deposited under code 3N5U.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

A.H., M.C., F.A.D. and S.M.R. all designed aspects of the study; A.H., M.C., R.C.S., M.R.S. and S.M.R. performed experiments; all authors analyzed data; F.A.D. and S.M.R. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein expression and purification. Recombinant PP1c (α isoform) was expressed in *E. coli* using a *tac* promoter; 2 mM $MnCl_2$ was added to the media upon induction. Purification was best achieved using a salt-dependent PP1c-inhibitor-2 association[46]. Full-length, human inhibitor-2 was expressed with an N-terminal hexahistidine tag in *E. coli*. Following cell lysis with 6 M urea, inhibitor-2 was bound to nickel sepharose beads and exchanged to a buffer containing 20 mM Tris, 50 mM NaCl, 15 mM imidazole, 0.4 mM $MnCl_2$, 0.2 mM TCEP and 0.1 mM PMSF (pH 8.0). Cells expressing PP1c were lysed in this same buffer, the cleared lysate was passed over the immobilized inhibitor-2 and PP1c was eluted with lysis buffer containing 1 M NaCl. For crystallography, PP1c was further purified with a Superdex75 column (GE Healthcare) equilibrated in 20 mM Tris, 500 mM NaCl, 2 mM DTT, 0.4 mM $MnCl_2$ (pH 8.0). RbC, $Rb_{55–928}$, E2F1-DP1 (RbC binding domains) and Cdk2-CycA protein constructs were expressed and purified as described previously[26,47,48]. The Rb constructs in both the calorimetry and kinetic experiments had N-terminal hexahistidine tags left intact. Cdk activating kinase from *Saccharomyces cervisiae* (Cak) was expressed as a GST fusion protein in *E. coli* and purified with glutathione sepharose affinity chromatography.

Isothermal titration calorimetry. ITC experiments were performed with a VP-ITC instrument (MicroCal). Typically, 0.5–1 mM of each RbC construct or synthetic RbC peptide was titrated into a 25–50 μM solution of PP1c. Experiments were carried out at 25 °C in a buffer containing 25 mM Tris, 100 mM NaCl and 1 mM DTT (pH 8.0). Each reported binding constant is the average from 2 or 3 experiments, and the reported error is the s.d. of the K_d from these measurements.

Crystallization and structure determination. Purified PP1c was concentrated to 10 mg ml^{-1} after the Superdex75 column and synthetic $Rb_{870–882}$ peptide (Biopeptide Co., Inc.) was added in a 3:1 molar ratio. Crystals were grown using the hanging-drop vapor diffusion method at room temperature (22 °C) . The crystallization buffer contained 100 mM HEPES, 200 mM $MgCl_2$ and 18% (w/v) PEG 4000 (pH 7.5) and was mixed in a 1:1 ratio with protein solution. Crystals grew with a needle morphology to dimensions of approximately 50 μm × 50 μm × 500 μm. Crystals were harvested by transferring to a solution containing 100 mM HEPES, 200 mM $MgCl_2$, 20% (w/v) PEG 4000 and 20% (v/v) glycerol (pH 7.5) and flash freezing in liquid nitrogen. A molecular replacement solution was obtained using the PP1c–microcystin crystal structure (PDB 1FJM) as a search model[34]. Further details regarding model building and refinement can be found in **Supplementary Methods**.

Phosphatase and kinase assays. Purified Cdk2-CycA was first activated by phosphorylation in a reaction containing 10% (w/w) GST-Cak, 10 mM $MgCl_2$ and 5 mM ATP. To prepare for the phosphatase assays, 1 mg of RbC was incubated with 0.25 mg of activated Cdk2-CycA for 1 h at room temperature in a buffer containing 50 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP and 5 μCi of ^{32}P-labeled ATP (pH 7.5). These reaction conditions give nearly quantitative phosphorylation of Cdk consensus sites in RbC[48]. Reactions were quenched by addition of 8 M urea, and phosRbC was isolated with a nickel-nitrilotriacetic acid spin column (Qiagen). Phosphatase reactions were carried out at room temperature in a buffer containing 50 mM HEPES, 100 mM NaCl and 2 mM $MnCl_2$ (pH 7.5). Reactions were initiated by addition of enzyme. Aliquots were removed at the indicated time point and quenched by mixing with SDS-PAGE loading buffer.

For the phosphatase steady-state analysis, dephosphorylation assays were carried out at varying substrate concentrations. The initial rate at each concentration was determined from a linear fit of band intensities of the first 3 or 4 time points, and the corresponding fitting errors were assigned as the error of each rate measurement. Initial rates were fit as a function of substrate concentration with a simple Michaelis-Menten model to calculate the effective K_M and k_{cat}.

For kinase assays, 75 nM activated (phosphorylated with CAK) Cdk2-CycA, 20 μM E2F1-DP1 and 2 μM RbC substrates were mixed in a reaction at room temperature containing the kinase buffer described above and 20 μCi of ^{32}P-labeled ATP (pH 7.5). In kinase reactions with PP1c, PP1c was first inactivated by mixing in a 1:3 molar ratio with L,R-microcystin. An additional 25 μM microcystin was present in the competition reactions to ensure no residual PP1c activity (the IC_{50} for microcystin is ~1 nM). Phosphorimaging was done with a Typhoon Trio gel scanner (Amersham) and data was analyzed with the ImageQuant software package (Molecular Dynamics). Kinetic data were fit with a first-order rate law using Kaleidagraph (Synergy Software). The reported error of each kinetic parameter is the fitting error.

Cell-cycle arrest experiments. We transfected 1 × 10^6 proliferating Saos-2 cells with 0.75 μg of CMV-Rb (0.5 μg in **Fig. 5b**), 1 μg of CMV-CD20 (used to mark transfected cells in flow cytometry analysis) and 6 μg of CMV-bGal, using Fugene 6 (Roche). Where indicated, 1 μg of CMV-HA-cdk2, 1 μg of CMV-CycA and 4 μg of CMV-myc-PP1c were added, and the appropriate amount of CMV-bGal was omitted to maintain uniform DNA concentrations. We analyzed cells 3 d following transfection by flow cytometry as described previously[49].

C33A cells were transfected with 10 μg of CMV-Rb, 2.5 μg of CMV-HA-cdk2, 2.5 μg of CMV CycA and 2.5 to 10 μg of CMV-myc-PP1c; CMV-βGal was included where necessary to obtain a final quantity of 25 μg. Transfections were performed by Ca_2PO_4 precipitation. Cells were harvested after 2 d, and nuclear lystates were prepared for SDS-PAGE and western blotting as described[50]. Rb was detected with monoclonal antibody G3-245 (BD Pharmingen) and anti-phosphoserine 807/811 antibodies from Cell Signaling.

For immunoprecipitation experiments, extracts were prepared as described above from Saos-2 cells transfected with Fugene HD (Roche). CV-1 cells were isolated by mitotic shakeoff from cultures that were first blocked in S phase with 2.5 mg ml^{-1} aphidicolin for 24 h then released for 16 h to enrich for mitotic cells. Immunoprecipitations were carried out using monoclonal antibody Rb4.1 (Developmental Studies Hybridoma Bank, University of Iowa) against Rb. Extracts and immunoprecipitated proteins were analyzed by SDS-PAGE alongside recombinant PP1c and GST-$Rb_{380–928}$ controls. Rb and PP1 were detected on western blots by Rb4.1 and sc-7482 (Santa Cruz), respectively. Standard curves to determine protein quantities were generated by using ImageJ software (US National Institutes of Health) to quantitate band intensities.

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Appendix B: List of plasmids

Name	Genes Encoded	Mutations	Obtained/Constructed	Res	Stock Number
pscodon-GST-p107-C	GST, p107(970-1069)	N/A	M. Cecchini	AMP	609
pscodon-GST-p130-C	GST, p130 (1040-1139)	N/A	M. Cecchini	AMP	610
pscodon-ΔL^{CRF} RBLP	GST, RBLP	Y756W	M. Cecchini	AMP	611
pscodon-ΔG-ΔL^{CRF}-RBLP	GST, RBLP	S463A, E464A, R544A, K548A, K652A, R656A, L660A, T664A, R668A, K873A, K874A, Y756W	M. Cecchini	AMP	612
pscodon-ΔGn-ΔL^{CRF}-RBLP	GST, RBLP	R467E, K548E Y756W	M. Cecchini	AMP	613
CMV-pRB-ΔSn	pRB	F839A	M. Cecchini	AMP	614
CMV-pRB-ΔGn-ΔSn	pRB	R467E, K548E, F839A	M. Cecchini	AMP	615
CMV-pRB-ΔGn-ΔL^{CRF}-pRB	pRB	R467E, K548E Y756W	M. Cecchini	AMP	616
CMV-pRB-ΔGn-ΔS-pRB	pRB	R467E, K548E M851A, V852A	M. Cecchini	AMP	617
CMV-ΔGn-ΔL^{CRF}-ΔS-	pRB	R467E, K548E M851A, V852A,	M. Cecchini	AMP	618

pRB		Y756W			
pflag-CMV-Skp2	Flag-Skp2	N/A	L. Zhu	AMP	619
Myc-PP1	Myc-PP1	N/A	T. Pawson	AMP	620
Myc-PP1-H248K	Myc-PP1	H248K	M.Cecchini	AMP	621
CMV-pRB-R876F-F877R	pRB	R876F, F877R	M.Cecchini	AMP	622
CMV-p27	p27	N/A	L. Zhu	AMP	623
CMV-p27 T127D	p27	T127D	L. Zhu	AMP	624
CMV-E2F1-Danio	E2F1	272-282 in human E2F1 replaces with *Danio rerio*	F. Dick	AMP	625
CMV-E2F1-Fugu	E2F1	272-282 in human E2F1 replaces with *Fugu rubripes*	F. Dick	AMP	626
pFAD 307 (E2F3-P329V)	E2F3	P329V	F. Dick	AMP	627
pFAD 309 (E2F1-Gallus)	E2F1	272-282 in human E2F1 replaces with *Gallus gallus*	F. Dick	AMP	629
pFAD 308 (E2F1-V276P)	E2F1	V276P	F. Dick	AMP	628
CMV-E2F1-V276A	E2F1	V276A	M. Cecchini	AMP	630
CMV-E2F3-	E2F3	P329A	M. Cecchini	AMP	631

P329A					
CMV-E2F2-P280V	E2F2	P280V	M. Cecchini	AMP	632
pRB-G Targeting Vector	*Rb1*	N/A	M. Cecchini	AMP	633
pBAC 252C16	*Rb1*	N/A	TCAG	Chlor	552
pscodon-RBLP	GST, RBLP	N/A	Fred Dick	AMP	519
pGEX-RBC	GST, RBC	N/A	Fred Dick	AMP	242
pFAD300	E2F1	N/A	Fred Dick	AMP	539
CMV-HA-E2F1	E2F1	N/A	Fred Dick	AMP	399
CMV-HA-E2F2	E2F2	N/A	J. Lees	AMP	319
CMV-HA-E2F3	E2F3	N/A	J. Lees	AMP	320
CMV-HA-E2F4	E2F4	N/A	J. Lees	AMP	318
CMV-CDK2	CDK2	N/A	s. van den Heuvel	AMP	345
CMV-CDK4	CDK4	N/A	s. van den Heuvel	AMP	101
CMV-DN-CDK2	DN-CDK2	N/A	s. van den Heuvel	AMP	342
CMV-Cyclin D	Cyclin D	N/A	P. Sicinski	AMP	96
CMV-Cyclin E	Cyclin E	N/A	J. Zhou	AMP	353
p73-Luc	Luciferase	N/A	C. Prives	AMP	496

pFAD102	pRB	N/A	Fred Dick	AMP	39
pFAD101	pRB	N/A	Fred Dick	AMP	37
CMV-TAg	Tag	N/A	B. Kennedy	AMP	98
CMV-β-Gal	β-gal	N/A	S. Salama	AMP	42
CMV-CD20	CD20	N/A	M. Classon	AMP	27
pE2F4B-Luc	Luciferase	N/A	F. Dick	AMP	138
p107(-280)-Luc	Luciferase	N/A	M. Classon	AMP	329
pBB14	GFP	N/A		KAN	348
CMV-Myc-Cdh1	Cdh1	N/A	N. Dyson	AMP	520
pUC19-RB-R467E-K548E	RB	R467E, K548E	M. Cecchini	AMP	560
pscodon-RBLP R467E-K548E	RBLP	R467E, K548E	M. Cecchini	AMP	561
CMV-RB-R467E-K548E	RB	R467E, K548E	M. Cecchini	AMP	562
pUC19-RB-K530D-K548E	RB	K530D, K548E	M. Cecchini	AMP	563
pscodon-RBLP K530D-K548E	RBLP	K530D, K548E	M. Cecchini	AMP	564
CMV-RB-K530D-K548E	RB	K530D, K548E	M. Cecchini	AMP	565

pscodon-RBLP R467E-K530D-K548E	RBLP	R467E, K530D K548E	M. Cecchini	AMP	566
pUC19-RB-R467E-K548E-ΔS	RB	R467E, K548E, M851A, V852A	M. Cecchini	AMP	567
pscodon-RBLP-R467E-K548E-ΔS	RBLP	R467E, K548E, M851A, V852A	M. Cecchini	AMP	568
pUC19-RB-K530D-K548E-ΔS	RB	K530D, K548E, M851A, V852A	M. Cecchini	AMP	569
pscodon-RBLP-K530D-K548E-ΔS	RBLP	K530D, K548E, M851A, V852A	M. Cecchini	AMP	570
CMV-E2F1-D277C	E2F1	D277C	M. Cecchini	AMP	571
pBABE	Puro	N/A	R. Hurford	AMP, Puro	28
pBABE-p16fl	p16, puro	N/A	J. Bruce	AMP, Puro	386
pBABE-p27	p17, puro	N/A	J. Bruce	AMP, Puro	390

Appendix C: PCR conditions

PCR Conditions *Rb1-ΔG*

Master Mix per reaction

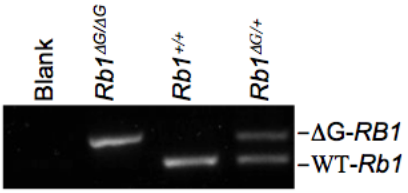
- 0.5µL $MgCl_2$
- 2µL 2mM dNTPs
- 2µL 10X PCR Buffer
- 1µL 20uM LoxP-N-F
- 1µL 20µM LoxP-N-R
- 13.5 µL Water
- 0.5µL Taq

- + 2µL DNA sample

Reaction Conditions

- MCGENO
  1. 94°C 2:00
  2. 94°C 0:45
  3. 60°C 0:45
  4. 72°C 0:45
  5. Go to Step #2, 35 times
  6. 72°C 7:00
  7. 4°C Forever

Interpretation of results



WT~250 b.p.

ΔG~ 330 b.p.

Primers

LOXP-N-F: ctgcaatctgcgcattttta

LOXP-N-R: cgatgctgcaggcctataat

PCR Conditions *Cdkn1b (p27)*

Master Mix per reaction

- 0.5 µL $MgCl_2$
- 2 µL dNTPs2 µL 10X PCR Buffer
- 0.5µL 20µM N1
- 2µL 20µM K3
- 2µL 20µM K5
- 8.5 µL Water
- 0.5µL Taq

- + 2µL DNA sample

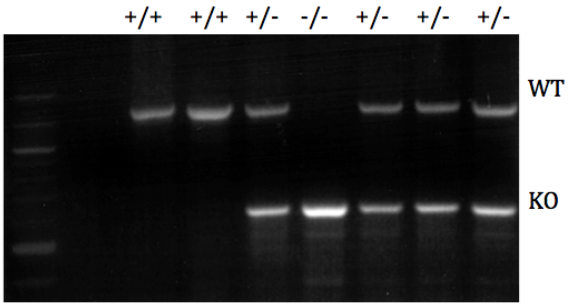
Reaction Conditions

- P27G
  1. 94°C 2:00
  2. 94°C 0:45
  3. 60°C 0:45
  4. 72°C 2:00
  5. Go to Step #2, 35 times
  6. 72°C 7:00
  7. 4°C Forever

Interpretation of result.

KO~600 b.p.

WT~ 1200 b.p.



Primer

K3: TGGAACCCTGTGCCATCTCTAT

K5: GAGCAGACGCCCAAGAAGC

N1: CCTTCTATGGCCTTCTTGACG

Appendix D: Antibody list

Antibody Name	**Protein recognized**	**Species**	**Supplier**	**Application***
3F10	HA-Epitope	Rat	Roche	WB
KH20	E2F1	Mouse	Santa Cruz	WB
PG37	E2F3	Mouse	Upstate	WB, IP
9E10	Myc-Epitope	Mouse	Hybridoma	WB, IP
G3-245	pRB	Mouse	BD pharmingen	WB
12CA5	HA-Epitope	Mouse	Hybridoma	WB, IP
PAb419	T-Ag	Mouse	Santa Cruz	WB
C36	pRB	Mouse	Hybridoma	WB
sc-7164	Skp2	Rabbit	Santa Cruz	WB
sc-6298	HDAC	Goat	Santa Cruz	WB
sc- 8272	RbAp46	Goat	Santa Cruz	WB
LY11	RBP1	Mouse	Hybridoma	WB
21C9	pRB	Mouse	Sibylle Mittnacht	ES
C-22	CDK4	Rabbit	Santa Cruz	ES
AN4.3	CDK2	Mouse	Upstate	ES
M-20	Cyclin E	Rabbit	Santa Cruz	ES
C-18	p107	Rabbit	Santa Cruz	WB, ES
C-20	p130	Rabbit	Santa Cruz	WB, ES
141.2	MCM7	Mouse	Santa Cruz	WB
pc10	PCNA	Mouse	Santa Cruz	WB
A2066	Actin	Rabbit	Sigma	WB

Rb4.1	pRB	Mouse	Hybridoma	WB, IP
PP1	sc-7482	Mouse	Santa Cruz	WB, IP
9308	Phospho-Rb (Ser807/811)	Rabbit	Cell Signaling	WB
347580	BrdU	Mouse	BD-Bioscience	IF, FC
347673	CD20	Mouse	BD-Bioscience	FC

*WB: Western Blot, IP: Immunoprecipitation, IF: Immunofluorescence,
ES: Electromobility shift, FC: Flow Cytometry

Appendix E: Permission Biochemical Journal

Data presented in chapter 2 is published in Biochemical Journal

Cecchini, M. J., and Dick, F. A. (2011) The biochemical basis of CDK phosphorylation independent regulation of E2F1 by the retinoblastoma protein, *Biochem J 434*, 297-308.

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Appendix F: Permission Nature Structural and Molecular Biology

Data presented in Appendix 1 is published in Nature Structural and Molecular Biology

Hirschi, A., Cecchini, M., Steinhardt, R. C., Schamber, M. R., Dick, F. A., and Rubin, S. M. (2010) An overlapping kinase and phosphatase docking site regulates activity of the retinoblastoma protein, *Nat Struct Mol Biol 17*, 1051-1057.

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Curriculum Vitae

Matthew Cecchini

Education

MD/PhD	Biochemistry	2014 (anticipated)	University of Western Ontario Supervisor: Dr. Fred Dick
hB.Sc	Biotechnology	2007	Brock University Supervisor: Dr. Jeffrey Atkinson

Awards and Accomplishments

- CaRTT trainee (2008-2012)
- CIHR MD/PhD Studentship (2007-2013)
- NSERC Canadian Graduate Scholarship (declined)
- Ontario Graduate Scholarship (declined)
- Distinguished Graduating Student Award (2007)
- Society of Chemical Industry Merit Award (2007)

Research and Professional Experience

- Graduate student in the laboratory of Dr. Fred Dick (2007- Present)
- Program advisory committee student representative for the Cancer Research and Technology Transfer program (2009-2010)
- Research Associate in the laboratory of Dr. Jeffrey Atkinson (2004- 2007)

Teaching Experience

- TA for third year protein and nucleic acid chemistry (2006-2007)
- TA for third year metabolic biochemistry (2006)
- TA for second year organic chemistry (2005-2006)

Publications

Pelka, P., Miller, M. S., **Cecchini, M**., Yousef, A. F., Bowdish, D. M., Dick, F., Whyte, P., Mymryk, J. S. (2011) Adenovirus E1A directly targets the E2F/DP-1 Complex, *J. Virol.* (*Accepted)*

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Cecchini, M., and Dick, F. A. (2010) E2F regulation is dispensable for growth control mediated by pRB. *The Cell Cycle: Cold Spring Harbor Laboratory.* (Oral Presentation)

Abstracts

Cecchini, M., and Dick, F. A. (2009) E2F and CdhI collaborate with pRB to regulate cell cycle entry. *The first international RB meeting*

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