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Characterizing the role of the Retinoblastoma protein LXCXE binding cleft in cellular senescence and tumor suppression

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

CHARACTERIZING THE ROLE OF THE RETINOBLASTOMA PROTEIN LXCXE BINDING CLEFT IN CELLULAR SENESCENCE AND TUMOR SUPPRESSION

(Thesis format: Integrated Article)

by

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Abstract

The Retinoblastoma protein (pRB) is a key regulator of the cell cycle and is functionally inactivated in most cancers. pRB has been proposed to utilize simultaneous interactions with E2F transcription factors and chromatin regulatory proteins to repress transcription and block cell cycle progression. The goal of this study is to characterize the physiological role of pRB interactions with chromatin regulatory proteins. I used gene targeted mice carrying point mutations in the murine *Rb1* gene ($Rb1^{\Delta L}$) that specifically disrupt pRB's LXCXE binding cleft, and thereby its ability to interact with chromatin regulatory proteins while leaving its ability to bind E2Fs intact. Embryonic fibroblasts from $Rb1^{\Delta L/\Delta L}$ mice fail to properly arrest DNA synthesis in response to senescence inducing signals such as oncogene expression and γ Irradiation. Failure to incorporate repressive heterochromatin marks like H3K9me3 results in de-repression of key cell cycle genes during senescence. However, this function of pRB is dispensable during normal differentiation and development, suggesting a specific role during stress responsive cell cycle arrest. Furthermore, during cellular senescence, pRB uses LXCXE binding cleft dependent interactions to recruit Promyelocytic leukemia protein (PML) to E2F target gene promoters. This function of pRB is important for establishment of heterochromatin marks and stable silencing of these genes thereby creating a permanent cell cycle arrest. Disruption of this function of pRB by the ΔL mutation confers susceptibility to escape from senescence and allows transformation *in vitro.* However, the same mutation does not enhance tumorigenesis in tumor models with activated ras mutations. $Rb1^{\Delta L/\Delta L}$ mice expressing oncogenic KrasG12D show delayed lung tumor formation compared to controls, which correlate with increased apoptosis in the early lesions following ras activation. Furthermore, DMBA treatment to induce ras mutations also fail to reveal greater susceptibility to cancer in $Rb1^{\Delta L/\Delta L}$ mice suggesting that loss of chromatin regulation by pRB has context dependent outcomes and does not universally enhance tumorigenesis *in vivo*.

Overall, this thesis enhances our current understanding of the unique role of pRB among the pocket proteins in cell cycle regulation by showing how pRB utilizes LXCXE binding cleft mediated interactions to stably block cell cycle in response to oncogenic stress signals.

Co-Authorship Statement

All chapters were written by Srikanth Talluri and edited by Dr. Fred Dick, with the exception of chapter 2, which was written by Dr. Fred Dick and edited by Srikanth Talluri.

All experiments in chapter 2 were performed by Srikanth Talluri with the following exceptions. Experiments in figures 2.1C & D were performed by Christian Isaac, and experiments in figures 2.2B & C were performed by Dr. Shauna Henley. Experiments in Figures 2.3 & 2.4 were performed by our collaborators Mohammad Ahmad and Dr. Rod Bremner. Dr. Sarah Francis performed experiments in figure 2.6. Alison Martens provided assistance with the mouse colony and helped with figure 2.5A.

All experiments in Chapters 3 were performed by Srikanth Talluri.

All experiments in Chapters 4 were performed by Srikanth Talluri with the exception of the experiments in Figure 4.6, which were done by Dr. Sarah Francis.

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Abbreviations

ATM: Ataxia telangiectasia mutated

ATR: ATM and Rad3-related protein

bp: Base pair

BSA: Bovine serum albumin

CDK: Cyclin dependent kinase

ChIP: Chromatin immunoprecipitation

CHK2: Checkpoint kinase 2

CKI: Cyclin dependent kinase inhibitor

CRF: Chromatin regulatory factor

DDR: DNA damage response

DMEM: Dulbecco's Modified Eagle Medium

DP: Differentiation Related Transcription Factor-1 polypeptide-1

E: Embryonic day

E2F: E2 promoter binding factor

FC: Flow cytometry

GSE: Gel shift extraction buffer

GST-p107-LP: GST tagged large pocket domain of p107

GST-RB-ΔL-LP: GST tagged large pocket domain of pRB with the ΔLXCXE mutation

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

GST: Glutathione S-transferase

HDFs: Human diploid fibroblasts

HP1: Heterochromatin protein 1

IF: Immunofluorescence

IP: Immunoprecipitation

kb: Kilo base

kD: Kilo dalton

LOH: Loss of heterozygosity

LSL-*KrasG12D*: LOX-STOP-LOX-*KrasG12D* mouse strain

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

MEFs: Mouse embryonic fibroblasts

mRNA: Messenger RNA

OIS: Oncogene induced senescence

p53: Human (*Tp53*) / mouse (*Trp53*) gene product

PBS: Phosphate buffered saline

PCNA: Proliferating cell nuclear antigen

PFA: Paraformaldehyde

PI: Propidium Iodide

ppRB: Hyper-phosphorylated pRB

pRB: Retinoblastoma tumor suppressor protein

RB1: Human retinoblastoma tumor suppressor gene

Rb1: Mouse retinoblastoma tumor suppressor gene

SAHF: Senescence associated heterochromatic foci

SA-β-gal: Senescence associated beta galactosidase

Tp53: Human tumor protein 53 gene

Trp53: Mouse transformation related protein 53 gene

WB: Western blot

X-gal: 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

ΔL: ΔLXCXE mutation

Table of Contents

List of Figures

List of Appendices

Chapter 1

1 Introduction

1.1 Cancer, and the discovery of the first tumor suppressor gene *RB1*

Cancer is uncontrolled cell division. Appropriate control of the cell division cycle is of utmost importance for tissue homoeostasis in multi-cellular organisms. Deregulation of this proliferative control is one of the major hallmarks of cancer (Hanahan and Weinberg, 2011). Ironically, much of our understanding of how a normal cell cycle is regulated has come from studying cancer. It led us to the discovery and understanding of the function of proto-oncogenes and tumor suppressor genes, the normal regulators of cell proliferation. This discovery of the genetic basis of cancer has transformed our understanding of the disease and greatly aided in the development of targeted therapies.

Retinoblastoma is a rare childhood cancer of the retina (Moll et al., 1997; Seregard et al., 2004). Retinoblastoma can occur as a unilateral disease, where only one eye is affected or can be presented as bilateral retinoblastoma where both the eyes are affected. Interestingly, the bilateral disease is very common in children with a family history of retinoblastoma. Alfred Knudson, in 1971, proposed a landmark theory of the genetic basis for retinoblastoma by using statistical analysis of the clinical data. He famously called it the "two hit hypothesis" (Knudson, 1971). According to this hypothesis, the generation of retinoblastoma requires two independent mutational events. Patients with familial retinoblastoma inherit one mutational event in their genome making them susceptible to retinoblastoma. A random second 'hit' can cause retinoblastoma in these individuals. Where as in individuals who do not inherit the first mutational event two independent mutations are required to cause retinoblastoma. Knudsen's hypothesis predicted the existence of a gene whose protein product suppresses retinoblastoma occurrence. Eventually, in 1986 two labs independently cloned the retinoblastoma gene 1(*RB1)*; the first tumor suppressor gene (Friend et al., 1986; Lee et al., 1987). Analysis of the *RB1* gene from retinoblastoma patients further confirmed Knudsen's hypothesis. Patients with familial retinoblastoma were in fact found to carry

one mutant allele of *RB1* in all their cells and they loose the remaining wild type allele as a result of loss of heterozygosity (LOH) (Cavenee et al., 1983) (Dryja et al., 1984).

1.1.1 The retinoblastoma 1 gene product (pRB) is a cell cycle regulator

Shortly after the cloning of the *RB1* gene, several viral oncoproteins (adenoviral protein E1A, Human papilloma virus protein E7, and Simian virus 40 T antigen) were shown to interact with the retinoblastoma 1 gene product, pRB (DeCaprio et al., 1988; Dyson et al., 1990; Dyson et al., 1989; Ludlow et al., 1989; Whyte et al., 1988; Whyte et al., 1989). All these oncoproteins were found to use a similar region to interact with pRB and this interaction is necessary for their ability to transform cells (Figge et al., 1988; Moran, 1988). At the same time, pRB was shown to contain properties of a cell cycle regulatory protein. pRB was shown to be phosphorylated in a cell cycle dependent manner in late G1 just before cells enter S-phase. This suggested that pRB might regulate the G1 to S phase transition in the cell cycle (DeCaprio et al., 1989). Finally, the last piece of the puzzle, the E2F transcription factor, was identified as a cellular target for pRB. pRB is shown to inhibit the transcriptional activity of E2Fs in the G1 phase of the cell cycle (Chellappan et al., 1991; Helin et al., 1992; Hiebert et al., 1992). E2F transcriptional activity peaks at the G1/S transition, which also corresponds with the hyper-phosphorylation of pRB by the cyclin/CDK complexes. Taken together, these studies led to the identification of a G1 checkpoint controlled by pRB through its interaction and inhibition of E2F transcription factors (Dyson, 1998).

1.2 The pocket protein family

Two pRB related proteins p107 and p130 were discovered based on their shared ability to interact with viral oncoproteins (Cobrinik et al., 1993; Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993; Zhu et al., 1993). They are encoded by *RBl1* (retinoblastoma like 1) and *RBl2* (retinoblastoma like 2) genes respectively. The p107 and p130 proteins share high structural homology with pRB and together they are often referred to as ‘pocket proteins’ (Classon and Dyson, 2001; Mulligan and Jacks, 1998).

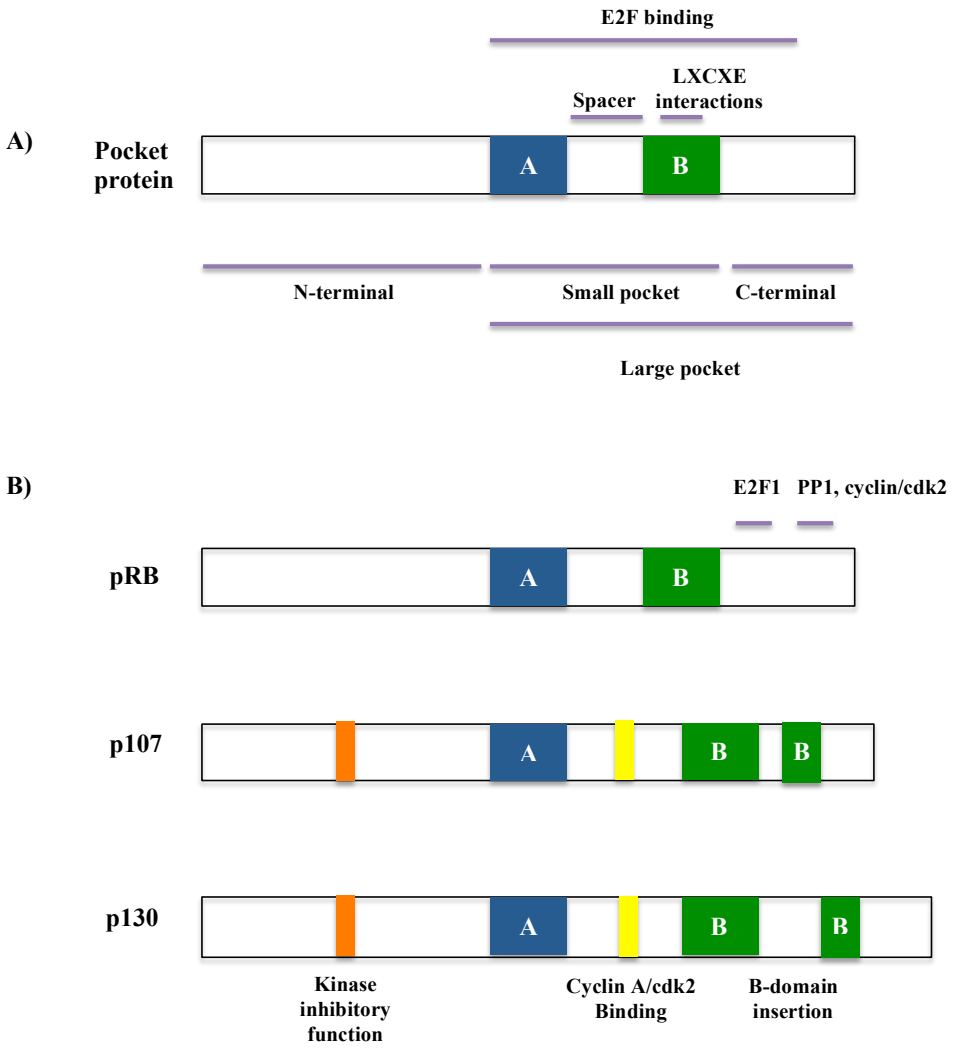

Figure 1.1 Domain structure of the pocket proteins

A) Domain structure highlighting the general features common to all the pocket proteins. B) Domain structure of pRB, p107 and p130 proteins highlighting their unique features.

The name ‘pocket protein’ is derived from the ‘pocket domain’ that is common to all three proteins in the family (Fig.1.1). The pocket region can be further divided into the small and large pocket. The small pocket consists of A and B domains separated by a spacer region. The A and B domains each form cyclin like folds that interact to form a globular pocket (Gibson et al., 1994; Lee et al., 1998). The small pocket is the minimal domain capable of interacting with viral oncoproteins (Kaelin et al., 1990). The large pocket region on the other hand, includes the ‘small pocket’ along with the C-terminal domain (Qin et al., 1992; Yang et al., 2002). The large pocket is required for E2F binding *in vivo* and is the minimal growth suppression domain in the pocket proteins.

One of the highly conserved features among the pocket proteins is the LXCXE binding cleft (Lee et al., 1998). The LXCXE binding cleft is the name used to describe the region of the pRB pocket that binds to the LXCXE peptide motif. The LXCXE motif was originally identified in viral oncoproteins such as adenovirus E1A, SV40 TAg and HPV-E7 that bind to the pocket domain of pRB. This peptide motif is necessary for the viral oncoproteins to bind pRB and transform cells (Kim et al., 2001). The crystal structure of the pRB A/B domain bound to the HPV E7 LXCXE peptide motif has identified a surface exposed cleft within the B region that binds to the LXCXE peptide (Lee et al., 1998). Interestingly, binding of the LXCXE peptide does not prevent the binding of an E2F peptide to the small pocket suggesting the possibility of a ternary complex (Lee et al., 1998).

The high sequence conservation of the LXCXE binding site residues, and the fact that this site is targeted for disruption by viral oncoproteins suggests that cellular interactions mediated by this cleft are key to pRB function. Over the years a number of cellular proteins have been identified that are found to interact with pRB through its LXCXE binding cleft (Dick, 2007). Interestingly, most of the proteins that bind to pRB through the LXCXE binding cleft are found to have enzymatic activities that can alter chromatin structure and act as co-repressors of transcription. These include proteins such as, histone deacetylases (HDAC1, HDAC2) (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998), histone demethylases (RBP2) (Benevolenskaya et al., 2005), DNA methyl transferases (DNMT1) (Robertson et al., 2000a), helicases (Brg1,

Brm) (Dunaief et al., 1994; Singh et al., 1995b), histone methyl transferases (Suv39h1, RIZ, and Suv4-20h1/h2) (Gonzalo et al., 2005; Nielsen et al., 2001; Steele-Perkins et al., 2001) and histone binding proteins like HP1 (Nielsen et al., 2001; Vandel et al., 2001).

Even though there is extensive sequence conservation among the three mammalian pocket proteins, there are a few subtle differences that separate them. In general, there are more similarities between p107 and p130 than between either of them and pRB. Both p107 and p130 contain B domain insertions. Furthermore, p107 and p130 also have a longer spacer region connecting the A and B domains (Lacy and Whyte, 1997; Woo et al., 1997; Zhu et al., 1995a). These long spacers allow them to stably interact with the cyclin/CDK complexes. Also, p107 and p130 contain a unique N-terminal domain that can inhibit cyclin dependent kinases (Woo et al., 1997).

pRB, also has some unique structural features mainly in the C-terminal domain that distinguishes it from p107 and p130. The pRB-C terminal fragment is uniquely capable of binding specifically to E2F1 (Dick and Dyson, 2003; Julian et al., 2008) and is implicated in regulating functions outside of normal cell cycle control. The C-terminal of pRB also contains binding sites for cyclin-CDK complexes and protein phosphatase 1 (PP1) (Adams et al., 1999; Durfee et al., 1993; Tamrakar and Ludlow, 2000; Vietri et al., 2006). Recently, a competitive interaction between cyclin/CDKs and protein phosphatase 1 (PP1) with pRB-C terminal was described suggesting a potential regulatory mechanism (Hirschi et al., 2010).

1.3 The E2F family of transcription factors

E2F transcription factors are a family of proteins that have important roles in cell proliferation in eukaryotes (Chen et al., 2009; DeGregori and Johnson, 2006). So far, eight mammalian E2Fs (1-8) have been identified (Fig. 1.2). E2F1, E2F2 and E2F3 are generally described as activator E2Fs due to their ability to strongly activate E2F transcriptional targets. E2Fs 4-8 are grouped under repressor E2Fs due to their ability to block E2F dependent transcription.

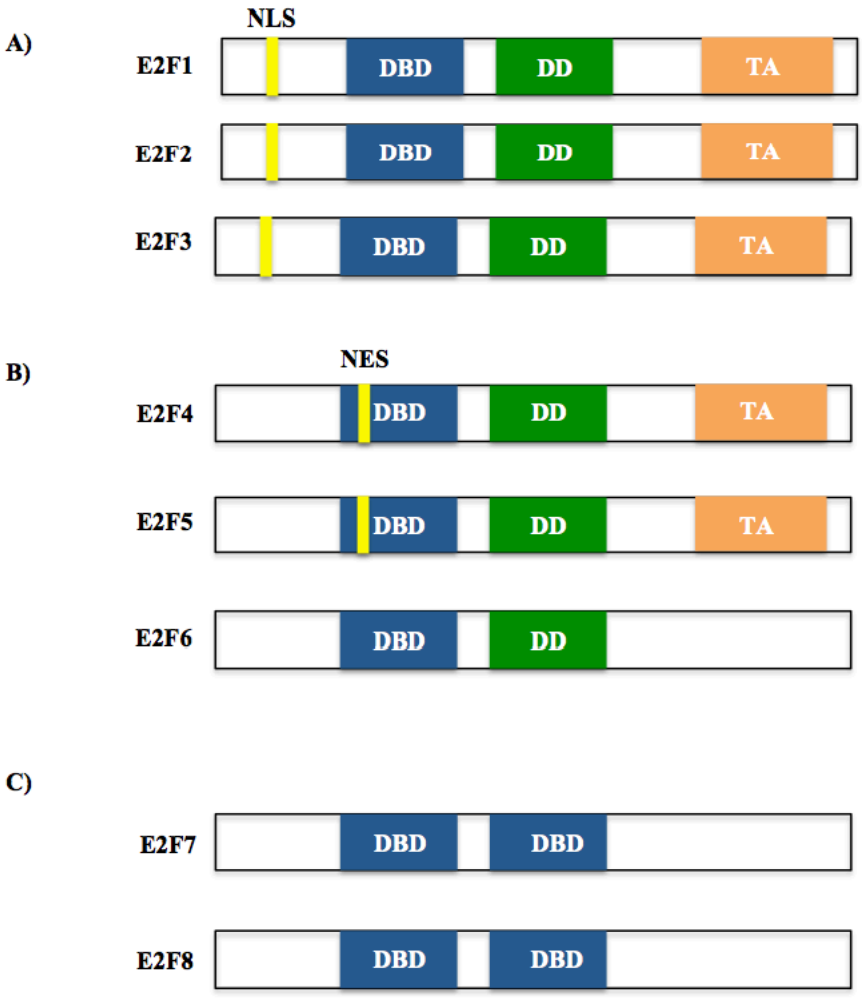

Figure 1.2 Domain structures of the E2Fs

The nuclear localization signal (NLS), the nuclear export signal (NES), the DNA binding domain (DBD), the dimerization domain (DD), and the transactivation domain (TA) are shown. (A) The activator E2Fs (B) The repressor E2Fs (C) The atypical E2Fs.

The pocket protein interaction motif in E2Fs is their transactivation domain. E2Fs were shown to have preferential binding to specific pocket protein partners. E2Fs 1-3 interact exclusively with pRB (Chen et al., 2009). E2F4 can interact with pRB but in general both E2F4 and E2F5 show preferential binding to p107 and p130 (Chen et al., 2009). E2F6, E2F7, and E2F8 lack a transactivation domain and do not interact with any of the pocket proteins.

E2Fs 1-6, all contain a DNA binding domain and a dimerization domain that is required for interaction with the DP proteins (Girling et al., 1993; Wu et al., 1995; Zheng et al., 1999). Three mammalian DP proteins (DP1, DP2/3, and DP4) have been identified so far. Hetero-dimerization with DP is critical for the function of E2F1-6 as they are not capable of interacting with DNA on their own. However, E2F7 and E2F8 have two DNA binding domains and can interact with DNA as homo or hetero-dimers independently of DP (Di Stefano et al., 2003; Maiti et al., 2005; Moon and Dyson, 2008).

1.4 Cell cycle regulation by the pocket proteins

The ability of the pocket proteins to regulate cell cycle is generally attributed to their ability to directly bind to and repress E2F transcription factors (Dyson, 1998; Hanahan and Weinberg, 2000; Trimarchi and Lees, 2002). Growth factor stimulation inactivates the pocket proteins through phosphorylation by cyclin dependent kinases (CDKs) releasing the E2F proteins to activate transcription of the cell cycle genes (Classon and Harlow, 2002a). Viral oncoproteins, like E1A, bind to the pocket proteins preventing them from interacting with E2Fs thereby inducing cell proliferation (Whyte et al., 1988; Whyte et al., 1989). Similarly, in cancer cells, the pRB pathway is inactivated either by direct mutation of the *RB1* gene, deregulation of CDKs, or inactivation of cyclin dependent kinase inhibitors such as $p16^{INK4A}$. These mutational events serve to stably deregulate E2F transcription (Sherr and McCormick, 2002) leading to uncontrolled cell proliferation.

However, apart from the negative regulation of E2Fs, pRB-E2F complexes were found to be capable of actively repress gene transcription from E2F promoters

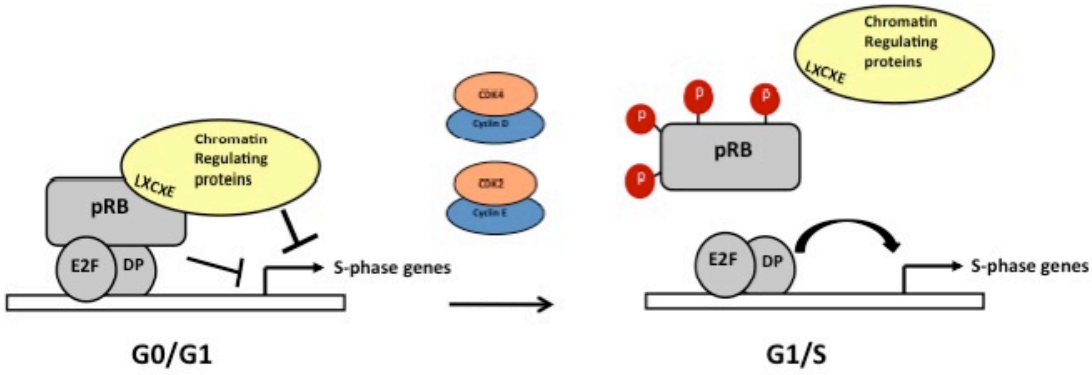

Figure 1.3 Current model of pRB function in G1/S phase transition

In G1, pRB interacts with the transactivation domain of E2F/DP heterodimer and blocks their activation of E2F target genes. pRB is capable of recruiting chromatin regulatory proteins to further repress the activation of these genes by generating a repressive chromatin environment. During G1 into S phase transition cyclin/Cdk complexes 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 rest of the cell cycle.

(Hamel et al., 1992; Weintraub et al., 1992). These observations suggested that pRB-E2F might be part of a bigger transcriptional repressor complex. In fact, pRB has been found to associate with a number of proteins that can regulate chromatin structure and repress transcription at E2F responsive promoters (Dick, 2007). These findings have expanded the model such that pRB is recruited to promoters by sequence specific transcription factors such as E2Fs. In turn, pRB recruits co-repressors such as HDACs through its LXCXE binding cleft to E2F responsive promoters. These chromatin regulatory factors modify chromatin in the neighboring regions and actively silence transcription (Fig. 1.3).

1.4.1 Overlapping and unique roles of individual pocket proteins

All the pocket proteins (pRB, p107 and p130) are in principle, capable of forming inhibitory complexes with E2Fs and recruiting co-repressors through the LXCXE binding cleft. However, several studies have found that specific pocket protein-E2F complexes predominate in different phases of the cell cycle and regulate the activity of E2Fs.

The differential activity of the pocket proteins seems partly due to their differential expression pattern during the cell cycle (Classon and Dyson, 2001). pRB expression is relatively unchanged throughout the cell cycle and it is expressed in both proliferating and non-cycling cells (Buchkovich et al., 1989). In contrast, p107 is an E2F target gene and its expression reflects free-E2F activity (Xiao et al., 1996; Zhu et al., 1995b). So, the expression of p107 is low in quiescent cells but increases sharply as cells enter S-phase. p130 expression is found to be very high in quiescent and terminally differentiated cells and low in proliferating cells (Cobrinik et al., 1993).

Our understanding of the distinct roles of the pocket proteins in development and cell cycle regulation has been greatly aided by the use of gene-targeted mouse models with specific disruption of individual pocket proteins. Disruption of the mouse *Rb1* gene results in embryonic lethality between E13.5 and E15 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The $Rb1^{-/-}$ embryos have increased proliferation and apoptosis in the nervous system and altered development and proliferation of the lens (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). However, many of the defects in $Rb1^{-/-}$ mice can be attributed to the proliferative defects in the placenta (de Bruin et al., 2003a). When

Rb1$^{-/-}$ mice were produced with normal placenta using tetraploid aggregation, many of the defects were rescued and these mice could survive until birth (Wu et al., 2003). However, they die shortly after birth with defects in skeletal muscle formation. This was found to be due to the failure of *Rb1*$^{-/-}$ myoblasts to terminally differentiate into multinucleated myotubes (Huh et al., 2004b). In addition, fibroblasts generated form *Rb1*$^{-/-}$ embryos (MEFs) also display proliferative defects in culture characterized by a shortened G_1 cell cycle phase and reduced cell size (Herrera et al., 1996b). They also fail to arrest properly in response to a variety of growth inhibitory signals such as TGF-β treatment , $p16^{INK4a}$ expression and DNA damage (Harrington et al., 1998; Herrera et al., 1996a; Medema et al., 1995).

On the other hand, p107$^{-/-}$ and p130$^{-/-}$ mice are viable, and normal (Lee et al., 1996). Combined disruption of both p107 and p130 however, results in lethality at birth, with severe defects in bone development (Cobrinik et al., 1996). This suggested that p107 and p130 have highly overlapping functions and can compensate for the loss of one another.

The relatively normal development of the *Rb1*$^{-/-}$ mice suggested that in some contexts p107 and p130 might compensate for the loss of pRB to maintain proliferative control. In fact, combined disruption of pRB and p107 or pRB and p130 resulted in early embryonic lethality between E11 and E13 (Dannenberg et al., 2004; Lee et al., 1996). Also, these embryos display more severe apoptotic and proliferative defects compared to the *Rb1* deletion alone. This suggested that the pocket proteins have partially overlapping functions and can compensate for the loss of other pocket proteins in certain contexts.

Triple knockout (TKO) fibroblasts lacking all the three pocket proteins were generated by differentiating targeted ES cells to further study the compensation among pocket proteins. These TKO fibroblasts were spontaneously immortal and showed defective arrest in the G1 under a variety of conditions (Dannenberg et al., 2000b; Sage et al., 2000b). Recently, TKO embryos lacking pocket proteins were successfully generated and these embryos survive until E9-E11. Some TKO cells were also found to be capable of exiting the cell cycle in G_1 and differentiating into epithelial and neural cell lineages

(Wirt et al., 2010). These surprising results suggested that G1 arrest and cell cycle exit could still occur in the absence of pocket protein activity in certain contexts.

Taken together, mouse models of pocket proteins have defined an essential role for pRB in mammalian development. pRB is required for placental development and muscle differentiation and proper proliferative control of certain tissues. On the other hand p107 and p130 have highly overlapping functions and play an essential role in bone development.

1.5 pRB's unique role in tumor suppression

In humans, loss of pRB results in the development of retinoblastoma early in life. Furthermore, *RB1* gene and/or components of the pRB regulatory pathway are mutated or silenced in most human cancers (Sherr, 1996; Sherr and McCormick, 2002). Surprisingly, a vast majority of the tumor-derived mutations identified in the pocket protein family are found in the *RB1* gene, suggesting a unique role for pRB among its siblings (Dick, 2007; Mulligan and Jacks, 1998).

Mouse models of pocket protein disruption also revealed pRB to be the major tumor suppressor among its family members. $Rb1^{+/-}$ mice develop tumors in the intermediate and the anterior lobes of the pituitary as well as medullary thyroid carcinomas (Harrison et al., 1995). In contrast, mice lacking p107 ($Rbl1^{-/-}$) or p130 ($Rbl2^{-/-}$) do not develop tumors in their lifetime (Classon and Harlow, 2002a). However, $Rb1^{+/-}$ mice do not develop retinoblastoma on their own and induction of retinoblastoma in mice requires disruption of both pRB and p107 (Chen et al., 2004b; Robanus-Maandag et al., 1998).

Taken together, these studies highlighted the unique role for pRB in tumorigenesis. However, the mechanistic basis for this unique tumor suppressive ability of pRB is still not known. Hence, an in depth analysis of the pRB tumor suppressor protein function is of great interest to cancer researchers.

1.6 A structure function approach to studying pRB function

One of the most intriguing features of pRB inactivation in human cancers is the rarity of missense mutant alleles. The vast majority of tumor derived alleles of *RB1* gene are deletions or non-sense mutations with only a very few missense changes (Valverde et al., 2005). In comparison, most cancer-derived mutations in the *TP53* gene target the DNA binding domain, suggesting its crucial role in p53 tumor suppressor function (Sherr and McCormick, 2002). Hence, relating pRB's proliferative control in culture to its tumor suppressive ability *in vivo* has not been possible from mutation data.

The discovery that viral oncoproteins (like E1A, TAg, and E7) and E2F transcription factors interact with the same region of pRB called the "pocket domain" has prompted investigators to map the functional domains of pRB. Cell culture assays, for pRB's role in proliferation and transcriptional repression identified the "large pocket" (aa379-928) as the minimal domain of pRB required for mediating these functions (Qin et al., 1992; Yang et al., 2002). However, several non-E2F cellular proteins have been discovered that interact with the large pocket in a manner similar to viral oncoproteins making it difficult to interpret how these numerous interactions control cell cycle progression (Dick, 2007). Structural analysis of the few available tumor derived missense mutations has shown that they non-specifically disrupt the over all protein structure, and as such, disrupt most of the interactions mediated by the large pocket (Dick, 2007).

The description of the crystal structure of pRB bound to a peptide derived from HPV E7 and similar structures of pRB bound to E2Fs in the recent years has allowed researchers to take a rational, structure guided approach to dissect the function of pRB (Burke et al., 2010; Lee et al., 2002; Lee et al., 1998; Rubin et al., 2005; Xiao et al., 2003). A number of discreet point mutations have been generated that specifically disrupt individual interactions within the large pocket without adversely affecting other pRB interactions (Dick and Dyson, 2003; Dick et al., 2000). These point mutants confirmed some of the previously proposed mechanisms of pRB function such as pRB-E2Fs in transcriptional control while revealing some novel functions of pRB outside of transcriptional regulation.

1.7 Gene targeted mice to study the contribution of the LXCXE binding cleft to pRB function

As stated previously, most of the proteins that bind to pRB through the LXCXE binding cleft have enzymatic activities that can alter chromatin structure and can act as co-repressors of transcription. The ability to bring these chromatin regulating activities to E2F responsive promoters allows pRB to influence broader genomic regions than just the DNA footprint of the E2F transcription factor.

In order to study the role that the chromatin regulator-pRB-E2F complexes play in cell cycle control, our lab and others have generated point mutants of pRB that specifically disrupt the LXCXE binding cleft (Dick, Sailhamer et al. 2000) (Fig. 1.4). Cell culture assays showed that this mutant of pRB is partially defective in transcriptional repression and cell cycle arrest (Chan et al., 2001). Furthermore, studies done in other labs have shown this mutant to be defective in its ability to mediate myogenic differentiation *in vitro* (Chen and Wang, 2000). These observations prompted our lab to investigate the physiological role of these interactions in an *in vivo* setting by introducing this mutant allele into the mouse germ line (Isaac et al., 2006a). We call this mutation RB-ΔLXCXE and the mice $Rb1^{\Delta LXCXE/\Delta LXCXE}$ or in short $Rb1^{\Delta L/\Delta L}$ mice. The $Rb1^{\Delta L/\Delta L}$ mice are viable, follow Mendelian inheritance, and develop relatively normally (Isaac et al., 2006a). This is in contrast to the $Rb1^{-/-}$ mice that die by embryonic day 15.5 due to proliferative defects in the placenta (Wu et al., 2003). This suggested that the LXCXE binding cleft interactions are dispensable for early embryonic development. Characterization of the $pRB^{\Delta L}$ protein in cells isolated from these mice revealed that it is expressed at similar levels to the wild type. As expected, $pRB^{\Delta L}$ is defective for interactions mediated by the LXCXE binding cleft, while retaining E2F binding. Importantly, unlike the $Rb1^{-/-}$ cells, the levels of the other pocket proteins (p107 and p130) are not elevated in the $Rb1^{\Delta L/\Delta L}$ MEFs, suggesting compensatory effects are unlikely (Isaac et al., 2006a).

Further characterization of the $Rb1^{\Delta L/\Delta L}$ mice, and cells derived from them, provided interesting insights into the physiological role of the pRB-LXCXE interactions.

ΔLXCXE mutation
I746A, N750A, M754A

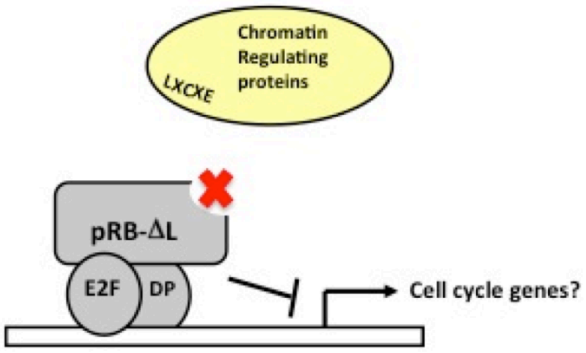

Figure 1.4 The RB-ΔLXCXE mutation

Disruption of the LXCXE binding cleft of pRB through three discreet amino-acid substitutions. The RB-ΔLXCXE mutation only disrupts the interactions mediated by the LXCXE binding cleft of pRB while leaving the other interactions such as E2F binding intact.

Rb1$^{\Delta L/\Delta L}$ females fail to nurse their pups and histological analysis showed epithelial hyperplasia in the mammary ducts (Francis et al., 2009b). A very interesting observationthat came from studying the *Rb1*$^{\Delta L/\Delta L}$ cells is that pRB has a novel role out side of G1 that is important for proper mitotic chromosome segregation and genome stability (Coschi et al., 2010). Strikingly, this non-G1 function of pRB is found to be tumor suppressive.

Studies done using the embryonic fibroblasts (MEFs) from *Rb1*$^{\Delta L/\Delta L}$ mice in terms of proliferative control provided surprising insights into the physiological role of the LXCXE type interactions. *Rb1*$^{\Delta L/\Delta L}$ MEFs show similar growth rate compared to wild type MEFs and exit from the cell cycle in response to confluence arrest and serum starvation. The transcriptional repression of E2F target genes also remains largely unaltered in these cells (Isaac et al., 2006a). This suggested that the recruitment of chromatin regulators by the pRB-LXCXE binding cleft is not essential for cell cycle gene repression and cell cycle exit in this paradigm of reversible growth arrest. However, it also raised a very interesting question of what physiological circumstances require the pRB-LXCXE interactions and whether this transcriptional repression function contributes to tumor suppression by pRB.

Strikingly, MEFs derived from the *Rb1*$^{\Delta L/\Delta L}$ mice show defective cell cycle arrest in response to a variety of stress stimuli such as the growth inhibitory cytokine TGF-β, CDK inhibitors such as p16^{Ink4a} and p21^{Cip1}. This suggested that chromatin regulation by pRB is required only under specific growth inhibitory contexts for the induction of cell cycle arrest. My work will expand on this concept in chapter 2 where I studied chromatin regulation by pRB in the context of cellular senescence (Talluri et al., 2010) & (Chapter 2). Cellular senescence is widely considered a key tumor suppressive mechanism that prevents the proliferation of precancerous cells from becoming tumorigenic. The next section will introduce cellular senescence and highlight the studies that show the key anti-proliferative and tumor suppressive properties of senescence. I will also highlight the current literature about pRB's role in senescence.

1.8 Cellular senescence

Cellular senescence can be defined as a stable cell cycle arrest in which cells exit the cell cycle and remain post-mitotic for an extended period of time (Campisi, 2005). Although, by definition, senescence seems very similar to terminal differentiation, these two stable cell cycle exit paradigms differ fundamentally in many ways. Whereas terminal differentiation is a developmentally regulated process that helps in functional specialization of cells, senescence is more of an aging and disease-associated phenotype. Senescence is proposed to be a stress responsive phenotype that curtails the growth and expansion of potentially deleterious cells and aids in tissue homeostasis.

1.8.1 Biomarkers of senescence

Senescent cells show a number of distinct morphological features as well as characteristic changes in gene expression and chromatin structure (Campisi and d'Adda di Fagagna, 2007a). However, there is no single reliable marker to identify senescent cells and a combination of different markers is commonly used to determine whether a cell population is senescent or not.

The most commonly used biomarker marker for senescence is the expression of a lysosomal enzyme called senescence associated β-galactosidase (SA-β-gal) (Dimri et al., 1995). In senescent cells the enzymatic activity of SA-β-gal increases at pH 6.0 and can be easily determined using a biochemical assay. The increased activity of SA-β-gal was later found to be a result of increased lysosomal content and activity in senescent cells (Kurz et al., 2000; Lee et al., 2006).

Another important biomarker for senescence is the cell cycle arrest. Senescent cells predominantly arrest in the G1 phase of the cell cycle (Sherwood et al., 1988). Senescent cells are also refractory to growth factor stimuli and can remain arrested for an indefinite period of time. The cell cycle arrest is normally determined by pulse labeling the cells with BrdU followed by flow-cytometric analysis. One other simple marker is the cell morphology. Cells undergoing senescence often display a large and flat morphology in cell culture. Apart from this, senescent cells also show increased number of vacuoles and increase in size of the nucleus and nucleolus.

Senescent cells show a distinctive gene expression pattern reflective of their phenotype. In general, they down regulate proliferative genes such as Cyclin E1, Cyclin A2, PCNA etc., and up regulate anti proliferative genes such as $p16^{Ink4a}$, $p19^{Arf}$, and $p21^{Cip1}$ (Campisi and d'Adda di Fagagna, 2007a) (Chicas et al., 2010). For this reason, increased expression of $p16^{Ink4a}$ and $p19^{Arf}$ proteins is also routinely used as a marker for senescence in cells and tissue sections.

1.8.2 Types of senescence

1.8.2.1 Replicative senescence

Senescence was originally identified as a phenomenon that limits the replicative life span of primary human diploid fibroblasts (HDFs) grown in culture (Hayflick and Moorhead, 1961). The authors of this study noticed that all the cell strains that they derived from a number of different fetal tissues undergo senescence after a finite number of divisions in culture. It has been shown later that this phenomenon is a result of gradual shortening of telomeres with each round of DNA replication (Wright and Shay, 2001). This is now commonly known as the Hayflick limit, or replicative senescence. Replicative senescence has since been observed in a number of different of cell types isolated from various tissues of all ages and also from cells derived from different species (Campisi and d'Adda di Fagagna, 2007a). All primary cultures irrespective of their origin undergo a finite number of divisions before they eventually senesce. Cell cultures are very heterogeneous and senescent cells can also be detected in young cultures even though at low numbers. The number of senescent cells in a culture progressively increases to a point where there is no further increase in the number of cells over time (Cristofalo and Sharf, 1973; Smith and Whitney, 1980).

1.8.2.2 Premature senescence

Surprisingly, a number of factors were later found to induce senescence in cultured cells independent of telomere length (Campisi, 2005; Dimri, 2005). Factors such as oxidative stress, γ irradiation and chemotherapeutic drugs induce senescence even in young cultures. This type of senescence is often called premature senescence. The number of factors that can trigger premature senescence is rapidly expanding. The

landmark finding that expression of activated oncogenes induces premature senescence in primary cultures has generated significant interest in studying the molecular mechanisms of senescence and its relevance to human disease (Serrano et al., 1997a). This along with a number of *in vitro* and *in vivo* studies over the last decade have placed senescence as a key tumor suppressive mechanism along with apoptosis that acts to prevent cancerous growth of damaged cells in response to a variety of stress signals (Campisi, 2001). Two such stress signals that are relevant to this thesis will be discussed in more detail in the following sections.

1.8.2.2.1 Senescence induced by oxidative stress in culture

Standard cell culture techniques involve culturing cells at 20% Oxygen (O_2). Human diploid fibroblasts (HDFs) grown under these conditions undergo approximately 50 population doublings before senescing. But, when HDFs were cultured at a more physiological oxygen levels of 3% O_2 they were able to undergo an additional 20 population doublings before senescing (Chen et al., 1995). The converse is also true where increasing the oxygen levels in culture to >20% resulted in early senescence (von Zglinicki et al., 1995). These studies showed that oxidative stress is one of the major inducers of premature senescence in culture. However, the sensitivity to oxygen levels varies considerably between cell types and between fibroblasts from different species. For example, mouse embryonic fibroblasts (MEFs) are more sensitive to oxygen levels in the culture medium and senesce approximately after 10-15 population doublings when grown at 20% O_2 (Parrinello et al., 2003). But, when cultured at 3% O_2 they grow for over 100 population divisions suggesting that oxygen sensitivity is a major driver of senescence in these cells (Parrinello et al., 2003).

1.8.2.2.2 Oncogene induced senescence (OIS)

Oncogene induced senescence (OIS) is the commonly used term to describe premature senescence induced by the expression of activated oncogenes in primary cells. Since the surprising discovery that expression of oncogenic HrasV12 induces senescence and a permanent exit from the cell cycle in primary cells (Serrano et al., 1997a), a number of different oncogenes have been reported to induce premature senescence,

suggesting that this is a common mechanism to counter oncogene induced transformation (Courtois-Cox et al., 2008).

How do oncogenes induce senescence? At first glance, it seems paradoxical that cancer causing oncogenes, as their name implies, activate senescence. Numerous studies in the past decade have tried to solve this apparent paradox and have advanced our understanding of how oncogenes elicit a senescence response (Di Micco et al., 2006; Mallette et al., 2007). When over expressed, activated oncogenes induce hyper proliferation, which puts an enormous stress on the cell's replication machinery. This slows the rate of replication fork progression and results in increased firing of replication origins and shortening of the inter-origin distance. This eventually leads to stalling of replication forks. Fork stalling leads to DNA double strand breaks and activation of the DNA damage response (DDR) characterized by phosphorylation and activation of ATM/ATR kinases and further down stream signaling through the p53 and pRB tumor suppressor pathways, the major effectors of the senescence response. Fibroblasts genetically lacking or depleted for key DDR proteins such as ATM and CHK2 fail to senesce in response to oncogene expression suggesting a crucial role for DDR in activating senescence. Furthermore, persistent DDR signaling seems to be essential for the maintenance of a senescent state in fibroblasts, because depletion of ATM in already senescent cells induces DNA replication and cell cycle re-entry.

DNA damage and activation of DDR seems to be the common denominator in senescence induced by most stimuli. In fact, replicative senescence, which is induced by shortening of telomeres, has also been shown recently to activate DDR (Abdallah et al., 2009). Critically shortened telomeres are identified as DNA breaks by cellular DNA damage sensing machinery resulting in the activation of senescence response.

1.8.3 Chromatin changes during senescence

Senescence is often associated with widespread changes in epigenetic modifications and heterochromatin organization. Human diploid fibroblasts such as IMR90, that are undergoing either replicative or premature senescence often display DAPI rich nuclear foci. These foci are now called senescence associated heterochromatic

foci (SAHF) (Narita et al., 2003a). A lot of our understanding of chromatin changes during senescence and their relevance to the senescence state has come from the study and characterization of these structures. SAHFs are devoid of active transcription and are highly enriched with repressive histone modifications such as H3K9me3 and H3K27me3 and heterochromatin protein1 (HP1) (Chandra et al., 2012; Narita et al., 2003a). In addition, activating histone modifications such as H3K9ac and H3K4me3 are often excluded from SAHFs. As the cells senescence, proliferative genes such as cyclin A become enclosed in these foci, a mechanism proposed to be important for stable silencing of these genes (Narita et al., 2003a).

Chromosome painting experiments showed that SAHFs are in fact individual chromosomes compacted into these DAPI dense bodies, highlighting global changes in the chromatin architecture during senescence (Funayama et al., 2006a; Zhang et al., 2005). A number of proteins have been reported that are either associated with SAHFs or are actively involved their formation. The histone variant macroH2A that is normally enriched in the inactive X chromosome gets incorporated into SAHFs (Zhang et al., 2005). The histone chaperone proteins Asf1a and HIRA are also necessary for SAHF formation and cell cycle exit during senescence (Zhang et al., 2007b; Zhang et al., 2005). Both these proteins are involved in the incorporation of the histone variant H3.3 into the nucleosomes suggesting that formation of SAHF might involve deposition of H3.3 (Galvani et al., 2008; Tagami et al., 2004).

Histone H1, the linker histone is lost in cells undergoing senescence and its loss also correlates with the ability of cells to form SAHFs (Funayama et al., 2006a). Surprisingly, the levels of high mobility group A (HMGA) proteins HMGA1 and HMGA2 increase during senescence (Funayama et al., 2006a; Narita et al., 2006). HMGA proteins are overexpressed in a number of cancers and are originally linked to transcriptional activation of a number of genes (Fusco and Fedele, 2007). It is interesting to note that HMGA proteins bind to the minor groove of AT rich DNA, which is also the binding site for linker histoneH1, prompting the suggestion that HMGA proteins replace Histone H1 on the DNA during senescence. Expression of HMGA1 or HMGA2 in primary human fibroblasts is sufficient to induce SAHF formation and senescence (Narita

et al., 2006). Conversely, knockdown of HMGA proteins impairs HrasV12 induced SAHF formation (Narita et al., 2006). However, the precise role of HMGA proteins in chromatin compaction and SAHF formation is still not clear.

The enrichment of H3K9me3 and HP1 protein in SAHFs suggested that these epigenetic changes might play a direct role in the formation of SAHFs (Narita et al., 2003a). It has been previously shown that recognition of H3K9me3 by the bromo-domain of HP1 protein and further recruitment of Suv39h1, the enzyme that tri-methylate's histone H3, aids in spreading of this repressive mark and formation of constitutive heterochromatin at the pericentromeric DNA (Bannister et al., 2001; Lachner et al., 2001). However, over expression of a dominant negative form of HP1β that drastically reduces the chromatin bound fraction of HP1 proteins failed to impair ras induced SAHF formation, suggesting that epigenetic changes and SAHF formation might be regulated independently during senescence (Zhang et al., 2007b; Zhang et al., 2007c). In fact, a recent study suggested that chromatin compaction into SAHFs and epigenetic modifications might occur independently of one another (Chandra et al., 2012). The authors over expressed JMJD2D demethylase that preferentially demethylates H3K9me3 or knocked down SUZ12, a component of PRC2 complex responsible for H3K27me3 to reduce the global levels of these repressive marks. Upon ras expression the H3K9me3 or H3K27me3 depleted cells still formed SAHFs to a similar extent as control cells (Chandra et al., 2012). This suggested that these epigenetic modifications are not a prerequisite for the formation of SAHFs.

A causal role for SAHFs during senescence has not been shown. However, senescence arrest in HDFs that form SAHFs is relatively more stable compared to the cells that do not form SAHFs during senescence suggesting that SAHFs might contribute to the long-term stability of senescent arrest by stably repressing the expression of proliferative genes (Beausejour et al., 2003; Narita et al., 2003a).

1.8.4 The *PML* gene and PML nuclear bodies in senescence

The Promyelocytic leukemia gene (*PML)* was originally identified in patients with acute Promyelocytic leukemia (APL). Leukemia cells from these patients harbor a

reciprocal chromosomal translocation t (15; 17) resulting in the fusion of the PML gene with retinoic acid receptor α gene (RAR α) (Piazza et al., 2001). The resulting PML-RAR α fusion protein is oncogenic and is sufficient to cause leukemia in transgenic mice (Piazza et al., 2001). The evidence for PML in tumor suppression comes from the analysis of tumorigenesis in *PML*$^{-/-}$ mice. *PML*$^{-/-}$ mice do not develop spontaneous tumors but are highly susceptible to carcinogenesis in response to chemical and physical stimuli such as DMBA treatment and ionizing (γ) radiation (Salomoni and Pandolfi, 2002). Crossing of PML-RAR α transgenic mice with *PML*$^{-/-}$ results in dramatic acceleration and increase of leukemia incidence, suggesting that inactivation of the tumor suppressive function of PML by the fusion protein might be a key step in AML pathogenesis (Rego et al., 2001).

PML is implicated in several biological processes such as growth suppression, differentiation, apoptosis, senescence and innate immunity (Jensen et al., 2001). The *PML* gene consists of 9 exons and alternative splicing generates multiple isoforms. The major PML isoforms are designated PML I-VII, which mainly differ in their C-terminal sequences (Fig. 1.5)(Jensen et al., 2001). The nuclear PML isoforms I-VI are essential components of the highly dynamic nuclear structures known as PML nuclear bodies (NBs). Over 30 cellular proteins co-localize with PML in these nuclear bodies and some of the proteins such as p53 and pRB physically interact with PML (Jensen et al., 2001). PML is also extensively modified by post-translational modifications and Sumoylation has been suggested to be important for NB formation (Seeler and Dejean, 2001). Most of the biological functions of PML have been linked to these nuclear bodies. The oncogenic PML-RAR α fusion protein disrupts these nuclear bodies and several viruses encode proteins that specifically disrupt the nuclear body formation further highlighting their importance (Dyck et al., 1994; Everett, 2001).

There are on average 5-15 NBs in most cells but their number and size dramatically increases in response to cellular stress such as viral infection, DNA damage, and aberrant oncogene expression (Salomoni and Pandolfi, 2002). Interferon is one of the best known inducers of PML expression (Chelbi-Alix et al., 1995; Regad and Chelbi-Alix, 2001). Recently, p53 has been shown to directly bind to the *PML* gene promoter

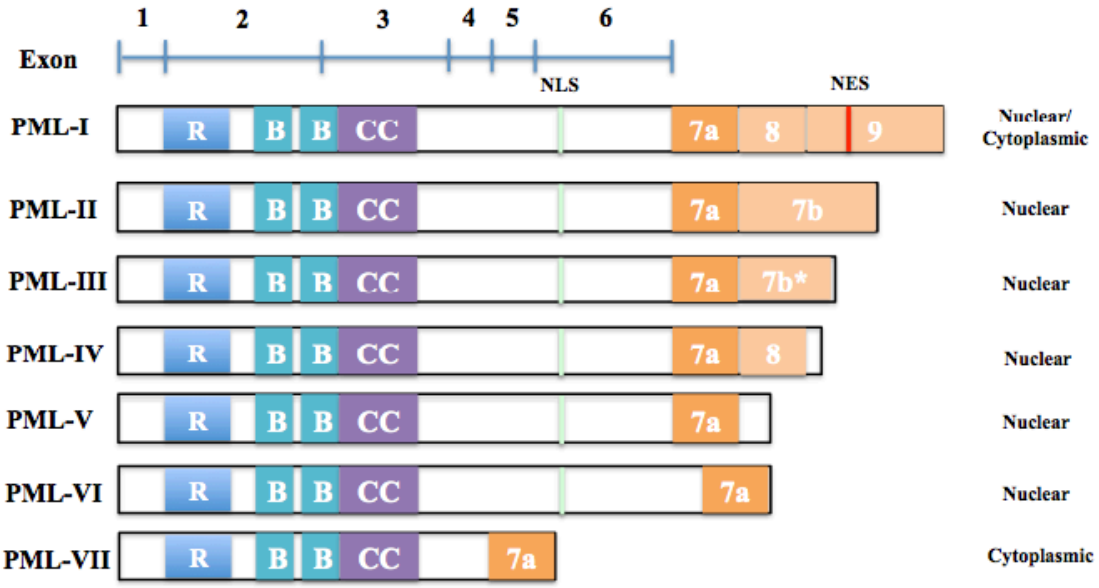

Figure 1.5 Domain structure of the PML isoforms

Alternative splicing of the C terminal region results in the translation of different PML isoforms. All isoforms contain the first three exons, which encode the RBCC motif, a tripartite motif that contains a zinc-finger RING domain (R), two zinc finger motifs (B- boxes) and a coiled-coil domain (CC). The RBCC domain promotes dimerization and the formation of the NB structures. The cellular localization of the isoforms is governed by the presence or absence of the nuclear localization signal (NLS) and nuclear export signal (NES) encoded by exon 6 and 9, respectively. Adapted from Nicola J.M. Brown et.al, Frontiers in Bioscience 14, 1684-1707, January 1, 2009.

and activate its transcription in response to oncogene expression and DNA damaging agents (de Stanchina et al., 2004).

The link between PML and senescence was discovered when it came up in genomic screens for genes up regulated in response to oncogenic HrasV12 expression in primary human fibroblasts (Ferbeyre et al., 2000). PML levels increase both during ras induced, and replicative senescence, and senescent cells show a dramatic increase in the number and size of the PML NBs. Over expression of PML itself can induce senescence in a p53 and pRB dependent manner depending on the cell type (Ferbeyre et al., 2000; Mallette et al., 2004). However, this feature is specific to PML-IV isoform suggesting its key role in promoting senescence among the isoforms (Bischof et al., 2002). Interestingly, over-expression of PML-IV fails to induce senescence in *PML*$^{-/-}$ MEFs suggesting that PML-IV alone is not sufficient for the induction of senescence and one or more of the other isoforms might play a role (Bischof et al., 2002). The key evidence for the essential role for PML in senescence comes from *PML*$^{-/-}$ MEFs which fail to senesce in response to oncogenic HrasV12 expression (Pearson et al., 2000).

1.8.5 Differences between human and mouse fibroblasts undergoing senescence

Mouse models and cells derived from mice are traditionally used as a tool to model human biology and to study the mechanism of human disease. Similarly, MEFs from knockout mouse models have been used to study senescence and its role as a tumor suppressor mechanism. As pointed out in the earlier sections, senescence mechanisms differ between cell types, cell strains and between species. The mechanism of senescence also depends on how a particular cell type responds to different stress signals.

Primary human cells undergo replicative senescence in culture after a certain number of population doublings (Hayflick and Moorhead, 1961). This is due to critical shortening of telomeres that triggers a DNA damage response leading to cell cycle arrest. But, mouse cells do not succumb to replicative senescence owing to their longer telomere DNA (30–150 kb versus 10 kb in humans) (Kipling and Cooke, 1990). However, mouse

cells are more sensitive to oxidative stress under standard cell culture conditions (20% O2) and senesce after ~10-15 population doublings (Parrinello et al., 2003).

Expression of oncogenic ras induces premature senescence in both mouse and human primary cells (Campisi, 2005). The p53 and pRB tumor suppressor pathways are the key regulators of cellular senescence in both human and mouse cells (Campisi, 2005). The p53 protein induces senescence partly by transcriptional activation of the cyclin dependent inhibitors (CKIs) such as p21. The pRB protein primarily blocks the cell cycle in G1 by inhibiting the transcriptional activity of E2F family proteins. The Arf-p53 pathway is considered to be the dominant pathway regulating senescence in mouse cells, compared to the p16/pRB pathway. MEFs from $p16^{Ink4a}$ null or $Rb1^{-/-}$ embryos enter at least a partial state of senescence in culture and in response to oncogenic stress (Krimpenfort et al., 2001; Sharpless et al., 2001; Sherr and DePinho, 2000) whereas MEFs from $p19^{Arf}$ *null* or $Trp53^{-/-}$ mice continue to proliferate under the same circumstances (Harvey et al., 1993; Kamijo et al., 1997). So, it has been suggested that the Arf–p53 pathway is the principal mediator of senescence in mouse cells. However, pRB/p107 double-knockout and pRB/p107/p130 triple knockout MEFs escape senescence in culture and are resistant to ras induced premature senescence (Dannenberg et al., 2000b; Peeper et al., 2001b; Sage et al., 2000b). Furthermore, acute depletion of pRB in MEFs is sufficient to reverse senescence suggesting its crucial role (Sage et al., 2003). These studies suggest that the p16/pRB pathway is also important for senescence in mouse cells.

Mouse embryonic fibroblasts (MEFs) unlike some human fibroblasts such as IMR90 do not condense their whole chromosomes to form SAHFs (Kennedy et al., 2010). Mouse cells under all growth conditions contain heterochromatin bodies in their nuclei that stain with DAPI and are called chromocentres (Probst and Almouzni, 2008). Chromocentres are pericentromeric heterochromatin regions that are rich in repetitive sequences. Whether mouse fibroblasts undergo global heterochromatin reorganization during senescence irrespective of the lack of visible chromosome condensation is still not clear and needs further investigation.

1.8.6 Oncogene induced senescence *in vivo*

Recent reports showed senescence in several mouse models of cancer both in response to oncogene activation (HrasG12V, KrasG12V, NrasG12D BrafV600E, Akt1 etc.,) and loss of tumor suppressors (Pten, pRB, Vhl etc.,) (Chen et al., 2005; Collado et al., 2005; Collado and Serrano, 2010; Dankort et al., 2007; Dhomen et al., 2009; Majumder et al., 2008; Sarkisian et al., 2007; Shamma et al., 2009; Young et al., 2008). In these mouse models the pre-malignant lesions such as lung adenomas and melanocytic nevi are often enriched in senescent cells. In contrast, senescent cells are rarely found in their corresponding malignant stages, such as lung adenocarcinomas and malignant melanomas suggesting a tumor suppressive role for senescence *in vivo*. Senescence is also observed in pre-malignant human lesions such as melanocytic nevi and Prostatic intraepithelial neoplasia (PIN) lesions suggesting its relevance to the human disease (Chen et al., 2005; Michaloglou et al., 2005).

Studies in several oncogene induced cancer models such as Nras induced B cell lymphomas, BrafV600E induced lung tumors and melanomas, and HrasG12V induced mammary tumors provided strong evidence for senescence as a tumor suppressive mechanism (Dankort et al., 2007; Goel et al., 2009; Sarkisian et al., 2007). Activation of oncogenes in these mouse models only resulted in a few small lesions associated with increased SA-β-gal activity, and markers of senescence. Interestingly, disruption of the regulatory pathways involved in oncogene induced senescence has been shown to result in increased susceptibility to tumorigenesis in these models. Simultaneous activation of oncogenes combined with loss of p53, p16 or Suv39h1, resulted in malignant transformation (Braig et al., 2005a; Chen et al., 2005; Sarkisian et al., 2007). Importantly, this is associated with the loss of senescent markers, strongly suggesting a tumor suppressive role for senescence in these models.

1.8.7 pRB's role in senescence

Early experiments in cancer cells lacking pRB suggested a critical role for it in senescence. Reintroduction of pRB into cancer cells that lack pRB induces senescence (Xu et al., 1997). Conversely, acute loss of pRB in senescent mouse embryonic fibroblasts (MEFs) results in increased DNA synthesis, cell cycle re-entry and subsequent

reversal of cellular senescence (Sage et al., 2003). *Rb1*$^{-/-}$ MEF's arrest in culture with features of senescence, but they escape from this arrest and immortalize sooner than control cells expressing the wild type protein (Dannenberg et al., 2000b; Sage et al., 2000b). These studies suggested a key role for pRB in establishing the stability of senescent cell cycle arrest.

The retinoblastoma protein appears to be capable of influencing senescence arrest at various levels. First, pRB represses the transcription of genes involved in DNA replication by directly binding to and inhibiting E2F transcription factors and through histone deacetylation of their respective promoters (Narita et al., 2003a). Indeed, pRB is found to be enriched on E2F target gene promoters during senescence (Chicas et al., 2010; Narita et al., 2003a) Acute knock down of pRB in primary human fibroblasts that are induced to senesce with oncogenic ras (HrasV12) show deregulated DNA synthesis as reflected in continued incorporation of BrdU and deregulated E2F transcription (Chicas et al., 2010).

Second, pRB is required for the enrichment of repressive histone methylation (H3K9me3) on E2F target gene promoters during senescence (Chicas et al., 2010). The precise mechanism of how pRB regulates the deposition of this repressive histone modification is still not known. However, this illustrates how pRB dependent chromatin regulation can exert its influence on transcriptional repression across E2F responsive promoters.

Lastly, pRB plays a role in the formation of SAHFs themselves, and thus also influences higher order chromatin structure in senescence as well. Knock down of pRB results in decreased formation of SAHFs (Chicas et al., 2010; Narita et al., 2003a). Since SAHFs represent the compaction of entire individual chromosomes (Funayama et al., 2006a; Zhang et al., 2005), these structures represent considerable reorganization of higher order chromatin structure that is pRB dependent. The exact signals that trigger this compaction, and the mechanisms of chromosome condensation that facilitates their formation are only beginning to be elucidated. Promyelocytic leukemia (PML) bodies appear to be a crucial component in the pathway to assembling SAHFs and they have

recently been shown to co-localize to genes that are silenced in a pRB dependent manner (Vernier et al., 2011). Again, it is unclear if pRB's actual function in the induction of chromosome condensation to form SAHFs mediates these events or if they are merely downstream of earlier pRB dependent steps.

Senescence, unlike other cell cycle exit paradigms, has distinguished itself as having a bona fide tumor suppressive role and pRB may need to use its full tumor suppressive ability in order to maintain the fidelity of this arrest. Since loss of pRB results in deregulated gene expression, DNA synthesis, and eventual escape from senescence, it is imperative that we investigate further the steps in gene silencing and higher order chromatin assembly that are controlled by pRB. In this way we will come to a thorough understanding of pRB function as a tumor suppressor protein.

1.9 Objectives of the present study

The LXCXE binding cleft is one of the highly conserved regions of pRB. The obvious suggestion from this observation would be that the function mediated by the LXCXE binding cleft is critical for the normal function of pRB. Despite a fair number of *in vitro* studies in cell lines using pRB mutants defective for the LXCXE interactions, the specific physiological contexts where these interactions are essential for pRB function as a tumor suppressor are not well known. The overall aim of this study is to characterize the role of the LXCXE binding cleft of pRB in cell cycle exit and tumor suppression using stress induced senescence as a physiological context.

First, I studied the ability of the $Rb1^{\Delta L/\Delta L}$ cells to permanently exit the cell cycle in response to developmental signals and stress stimuli that are potentially oncogenic. I hypothesized that the LXCXE binding cleft has a specific role in establishing permanent exit from the cell cycle in response to oncogenic insults. So, I tested this hypothesis using two modes of permanent cell cycle exit, terminal differentiation and cellular senescence. The results are discussed in detail in chapter 2 of the thesis.

In chapter 3 I further explored the role of pRB-LXCXE binding cleft mediated interactions during cellular senescence. I show how the $Rb1^{\Delta L}$ mutation disrupts the

ability of pRB to stably repress the transcription of cell cycle genes during cellular senescence. I further characterize the proteins that cooperate with pRB in a LXCXE dependent manner to maintain the stability of senescence arrest in primary mouse fibroblasts.

Finally in chapter 4, I tested the effect of $Rb1^{\Delta L}$ mutation on transformation potential in $Rb1^{\Delta L/\Delta L}$ cells both *in vitro* and *in vivo*. I used *in vitro* immortalization and transformation assays to test the ability of $Rb1^{\Delta L/\Delta L}$ cells to escape senescence. *In vivo,* I used an oncogenic Kras driven tumor model to study the effect of the $Rb1^{\Delta L}$ mutation on senescence induction and tumorigenesis in the lung.

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Chapter 2

2 A G1 checkpoint mediated by the retinoblastoma protein that is dispensable in terminal differentiation but essential for senescence

2.1 Abstract

Terminally differentiated cell types are needed to live and function in a post-mitotic state for a lifetime. Cellular senescence is another type of permanent arrest that blocks proliferation of cells in response to genotoxic stress. Here we show that the retinoblastoma protein (pRB) uses a mechanism to block DNA replication in senescence that is distinct from its role in permanent cell cycle exit associated with terminal differentiation. Our work demonstrates that a subtle mutation in pRB, which cripples its ability to interact with chromatin regulators, impairs heterochromatinization and repression of E2F responsive promoters during senescence. In contrast, terminally differentiated nerve and muscle cells bearing the same mutation fully exit the cell cycle and block E2F responsive gene expression by a different mechanism. Remarkably, this reveals that pRB recruits chromatin regulators primarily to engage a stress-responsive G1 arrest program.

2.2 Introduction

Terminal differentiation is fundamental to the development of a multicellular organism (Buttitta and Edgar, 2007). Of particular importance is the commitment to permanently exit the cell cycle. Many cells enter a post-mitotic state early in life and must remain viable and non-proliferative throughout the lifespan of the organism. Cellular senescence is another form of proliferative control that can be induced as a natural consequence of aging, or prematurely in response to stimuli such as DNA damage (Campisi and d'Adda di Fagagna, 2007b). The physiological differences between terminal differentiation and senescence suggest that there may be differences in their mechanisms of growth control, however, the robust control of cell cycle entry is an obvious similarity. Comparisons between the two are rare in current literature.

Coupling cell cycle exit with terminal differentiation requires the coordinated activities of the retinoblastoma (RB) family of proteins and cyclin dependent kinase (CDK) inhibitors (Buttitta and Edgar, 2007). Studies in organisms such as Drosophila and *C. elegans* support a general model in which cell cycle exit requires simultaneous regulation of E2F transcription by RB family proteins and cyclin/CDK activity by their inhibitors (Boxem and van den Heuvel, 2001; Buttitta et al., 2007; Firth and Baker, 2005). While it is unclear how this regulation is coordinated, it has been speculated that changes in chromatin structure could offer an explanation. Under this interpretation, heterochromatinization of cell cycle promoters blocks cyclin/CDKs from activating transcription through E2Fs, likewise promiscuous E2F activity is unable to induce expression of cyclins. For these reasons much attention has been focused on chromatin regulation in transcriptional control by pRB and this function has been reviewed extensively (Burkhart and Sage, 2008; Classon and Harlow, 2002b; Korenjak and Brehm, 2005). Cell cycle exit during terminal differentiation of neurons and skeletal muscle requires pRB function (Chen et al., 2004a; de Bruin et al., 2003b; Ferguson et al., 2002; Huh et al., 2004a; MacPherson et al., 2004; Zhang et al., 2004). In addition, deposition of heterochromatin at E2F responsive cell cycle promoters is reported to be pRB dependent during differentiation of these same cell types (Blais et al., 2007; Panteleeva et al., 2007). Unfortunately, efforts to uncouple cell cycle exit from differentiation through loss of pRB have been complicated because this often leads to cell death, particularly in muscle development (Camarda et al., 2004; Huh et al., 2004a; Zacksenhaus et al., 1996). This raises the question of whether chromatin regulation by pRB is the cause of cell cycle exit, or a consequence of differentiation.

Cell cycle exit in senescence also involves the coordinated action of CDK inhibitors and RB family proteins (Peeper et al., 2001a; Serrano et al., 1997b). However, the frequent participation of p53 in the induction of senescence distinguishes it from cell cycle exit in differentiation (Campisi and d'Adda di Fagagna, 2007b; Collado et al., 2007). In this cell cycle arrest paradigm, pRB has a central role in the generation of senescence associated heterochromatic foci (SAHF) (Narita et al., 2003b). SAHFs are single chromosomes compacted into microscopically visible heterochromatin bodies (Funayama et al., 2006b; Zhang et al., 2007a). This compressed genomic structure

ensures efficient silencing of E2F regulated cell cycle genes. Thus, pRB function is critical to establishing one of the features of senescence that best defines its permanence. However, not all senescent human fibroblasts form SAHFs (Funayama et al., 2006b). Fibroblasts from knock out mice have been used extensively to genetically dissect the pathway that induces senescence and this analysis has demonstrated that it requires RB family proteins (Dannenberg et al., 2000a; Peeper et al., 2001a; Sage et al., 2000a). Interestingly, the presence of SAHFs in senescent mouse cells remains in question because pericentromeric heterochromatin bodies are present under all growth conditions. Because not all senescent cells contain SAHFs, it is unclear whether pRB regulates chromatin structure in senescence in their absence.

Despite these gaps in our knowledge, regulation of chromatin structure by pRB is frequently linked with its function in cell cycle control (Burkhart and Sage, 2008; Classon and Harlow, 2002b). Many reports have shown that chromatin regulating enzymes such as Brg1 (Dunaief et al., 1994), Brm (Singh et al., 1995a), HDAC1 (Magnaghi et al., 1998), DNMT1 (Robertson et al., 2000b), and Suv39h1 (Nielsen et al., 2001) among others, use a peptide motif called LXCXE to interact with the pocket domain of pRB (Classon and Harlow, 2002b; McClellan and Slack, 2007). Through the simultaneous interaction with E2F transcription factors, this complex is recruited to E2F target genes to block transcription and arrest the cell cycle in G1 (Burkhart and Sage, 2008). In this way, a one-size-fits-all model of pRB has emerged in which this E2F-pRB-chromatin regulating repressor module is activated under all G1 cell cycle arrest circumstances to remodel chromatin and block proliferation. However, it is noteworthy that reports investigating the myriad of chromatin regulators that interact with pRB have largely been carried out using cell culture assays and this has prevented us from truly understanding the biological significance of chromatin remodeling by pRB. At present it is unclear if induction of senescence or terminal differentiation invokes the same pRB functions, even though they both can lead to a permanent G1 arrest that is frequently characterized by changes in chromatin structure.

To investigate how the recruitment of chromatin regulating activities by pRB influences mammalian development and disease, we have generated a gene-targeted

mouse strain in which mutations in pRB disrupt only LXCXE dependent interactions (Isaac et al., 2006b). We have validated that this mutation (called $Rb1^{\Delta L}$) disrupts numerous interactions between chromatin regulators and pRB, but leaves interactions with E2Fs intact (Isaac et al., 2006b). Importantly, the $Rb1^{\Delta L}$ allele expresses pRB at levels equivalent to wild type and the expression of the related RB-family proteins p107 and p130 are unchanged (Isaac et al., 2006b). This suggests that defects in $Rb1^{\Delta L/\Delta L}$ are not suppressed by over expression of other family members as is the case for $Rb1^{-/-}$ mice (Hurford et al., 1997; Mulligan et al., 1998). Despite the interactions that are disrupted, $Rb1^{\Delta L/\Delta L}$ knock in mice are viable (Isaac et al., 2006b), raising the question of what physiological circumstances require pRB to use chromatin regulation in cell cycle control?

In this report we compare the cell cycle exit properties of skeletal muscle and retinal neurons, two long-lived cell types, with senescent cells derived from $Rb1^{\Delta L/\Delta L}$ mice. Our work shows that there is defective inhibition of DNA replication in senescent $Rb1^{\Delta L/\Delta L}$ cells, but not in permanent cell cycle exit during development. This indicates that one of the primary functions of chromatin regulation by pRB is an arrest checkpoint that is used during senescence. The defect in senescence is a failure to create a repressive chromatin structure at E2F responsive genes and is characterized by a deficiency in H3K9me3. Conversely, ChIP analysis of the same promoters in $Rb1^{\Delta L/\Delta L}$ muscle reveals a different transcriptional silencing pathway characterized by a combination of H3K27me3 and H3K9me3 modifications that are present in normal abundance in $Rb1^{\Delta L/\Delta L}$ mutants. Unexpectedly, this reveals that pRB possesses a stress-responsive growth control mechanism that is distinct from cell cycle exit in terminal differentiation during development.

2.3 Results

2.3.1 The $Rb1^{\Delta L}$ mutation causes defects in a senescent cell cycle arrest

Based on pRB's well known role in controlling G1 to S-phase progression, we surveyed the ability of fibroblast cells from $Rb1^{\Delta L/\Delta L}$ mutant mice to respond to DNA

damaging agents, activated oncogenes, and other stimuli that are known to impinge upon proliferative control by pRB. Consistent with the discrete nature of the knock in mutation some growth arrest mechanisms worked normally, such as serum deprivation for 3 to 5 days (Fig. 2.1A). However, a number of senescence inducing stimuli like γ-irradiation and oncogenic ras were unable to generate a complete cell cycle exit in *Rb1*$^{\Delta L/\Delta L}$ fibroblast cells despite the fact that the cells ceased to divide and assumed a senescent morphology characterized by SA-β-Gal staining (Fig. 2.1B). *Rb1*$^{\Delta L/\Delta L}$ MEFs showed a normal response to DNA damage during the first 48 hours following γ-irradiation and largely ceased to incorporate BrdU (Fig. 2.1C). However, even at this early time point, reduced accumulation of cells in G1 became apparent (Fig. 2.1C). A similar analysis of DNA content five days post treatment revealed a striking failure of mutant cells to collect in G1 with many cells exhibiting abnormally high DNA content at 8N and beyond, indicative of endoreduplication (Fig. 2.1D). This occurred regardless of whether the arrest was induced by γ-irradiation or activated ras. Furthermore, 10 days following the induction of senescence by HrasV12, mutant MEFs still had elevated levels of BrdU incorporation relative to wild type controls (Fig. 2.1E). This suggests that persistent, but low levels, of DNA synthesis leads to the elevated DNA content found in *Rb1*$^{\Delta L/\Delta L}$ MEFs following the induction of senescence. We interpret this phenotype to mean that *Rb1*$^{\Delta L/\Delta L}$ cells are capable of entering a senescent state based on morphology, the presence of SA-β-Gal staining, and the inability to undergo mitosis. However, the mutation in pRB prevents an irreversible withdrawal from the cell cycle that allows endoreduplication. For these reasons, we will refer to the state of these cells as defective, or incomplete, senescence throughout this report.

2.3.2 Permanent cell cycle exit during development is normal in *Rb1*$^{\Delta L/\Delta L}$ mice

Because cellular senescence is thought to be an irreversible arrest, we decided to examine cell cycle exit and differentiation in long-lived cell types that remain growth arrested throughout life. A number of tissues possessing permanently arrested cells that fit this description are also known to require pRB for cell cycle exit during terminal differentiation. In particular, pRB has a well recognized role in cell cycle control of

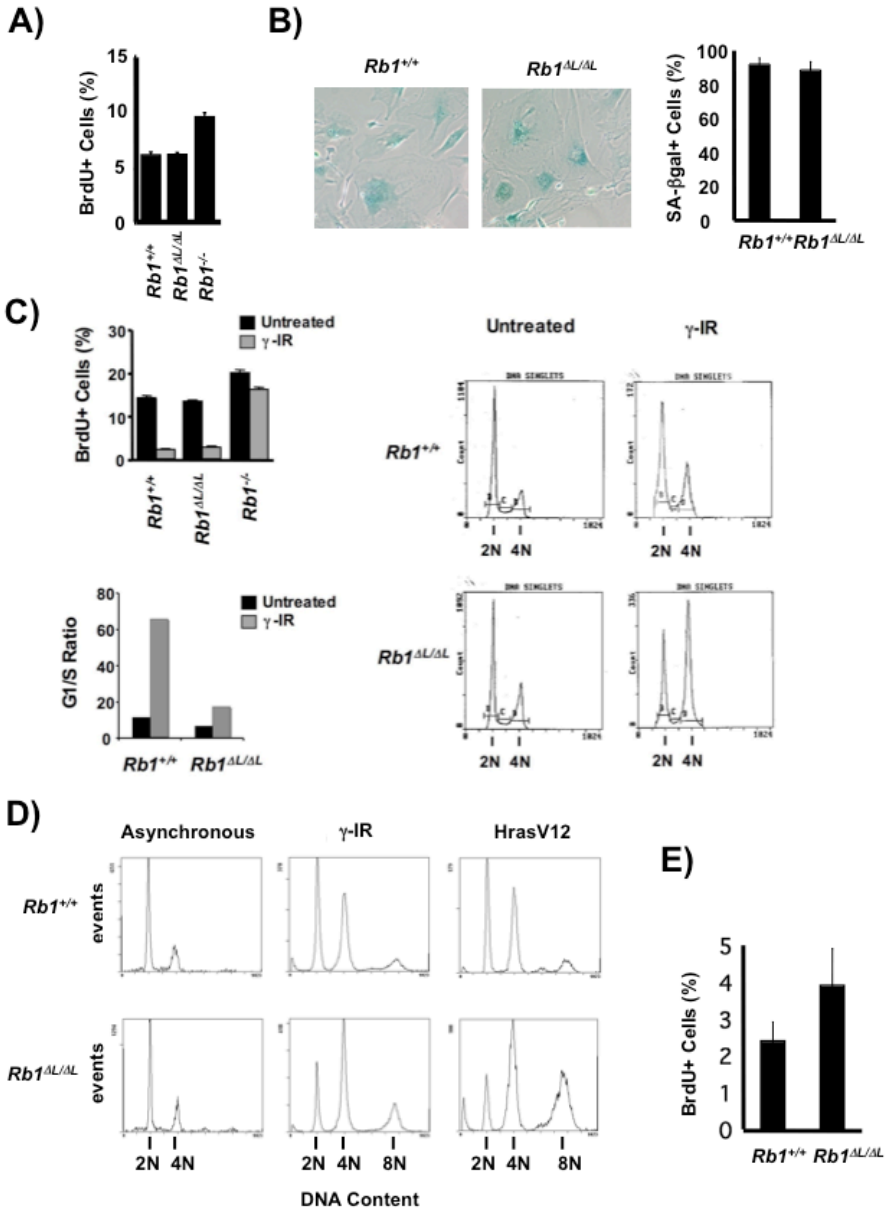

Figure 2.1 Defective arrest of DNA synthesis in *Rb1* mutant cells during senescence

(A) MEFs were serum deprived for 72 hours and DNA synthesis was measured by BrdU incorporation. (B) Ten days following retroviral transduction of MEFs with HrasV12, senescent cell morphology and SA-β-Gal activity were examined by light microscopy and the % SA-β-Gal positive cells is displayed in the graph to the right. (C) MEFs were irradiated with 15 Gys of radiation. 48 hrs later, cells were pulse labeled with BrdU and processed for PI/BrdU flow cytometry analysis. Quantification of the ratio of %G1 cells and %S phase cells from PI/BrdU analysis reveals that the G1 checkpoint in $Rb1^{\Delta L/\Delta L}$ mutant cells is defective. (D) DNA content of wild type and mutant MEFs was examined by PI staining and flow cytometry 5 days following irradiation or viral infection to express HrasV12. Numbers above the peaks indicate % nuclei with respective DNA content (E) DNA synthesis in senescent MEFs was measured by BrdU incorporation over eight hours, 10 days following viral infection. Error bars in all graphs indicate one standard deviation from the mean of at least three replicates. *P* value for t-test comparing mean measurements in E is 0.04.

muscle (de Bruin et al., 2003b; Zacksenhaus et al., 1996), as well as the retina (Chen et al., 2004a; MacPherson et al., 2004; Zhang et al., 2004), and we have examined the effects of the $Rb1^{\Delta L}$ mutation in these contexts.

When the placental defects of $Rb1^{-/-}$ mice are complemented, knock out animals die at birth with defects in myogenesis and are characterized by gross histological abnormalities and numerous apoptotic cells (de Bruin et al., 2003b). The fact that $Rb1^{\Delta L/\Delta L}$ mice are viable and appear normal suggests that pRB's role in muscle differentiation is complemented by the $Rb1^{\Delta L}$ allele (Fig. 2.2A). Indeed, histological analysis of skeletal muscle stained with Haematoxylin and Eosin (H & E) from $Rb1^{\Delta L/\Delta L}$ mutant mice reveals that they are indistinguishable from wild type controls (Fig. 2.2B). Beyond the ability of the $Rb1^{\Delta L}$ mutant to function in the differentiation of muscle, we also investigated the permanence of cell cycle exit in this tissue. Anti-BrdU staining demonstrates infrequent proliferation in cross sections of wild type and $Rb1^{\Delta L/\Delta L}$ muscle fibers, less than one per microscopic field of view (Fig. 2.2C). The quantity of rare, positively stained nuclei is consistent with proliferation of myosatellite cells that repair post-mitotic muscle fibers. From this analysis, ectopic DNA replication in myotubes appears to be absent. As a control for our ability to sensitively detect DNA replication, we also stained highly proliferative cells from intestinal crypts in the same mice to confirm that our labeling and staining robustly detect DNA replication (Fig. 2.2C). This analysis of cell proliferation in the muscle of $Rb1^{\Delta L/\Delta L}$ mice indicates that cells exit the cell cycle and remain post-mitotic in a manner comparable to wild type. In order to test if the transcriptional silencing function of pRB is intact in differentiated muscle of $Rb1^{\Delta L/\Delta L}$ mice, we studied the expression of E2F target genes like *Pcna*, *Ccne1* (cyclin E), *Rbl1* (p107), *Ccna2* (cyclin A), and *Tyms* (thymidylate synthase) (Fig. 2.2D). We found equal expression of these genes between wild type and mutant muscle. Western blots also showed similar levels of protein expression among E2F targets across the two genotypes, further suggesting that control of gene expression is properly maintained in $Rb1^{\Delta L/\Delta L}$ muscle (Fig. 2.2D). Importantly, this also reveals that expression of the related pRB family protein, p107, remains normal under these conditions. This indicates that myogenesis in $Rb1^{\Delta L/\Delta L}$ mutants is likely not the result of compensation by other pRB family members.

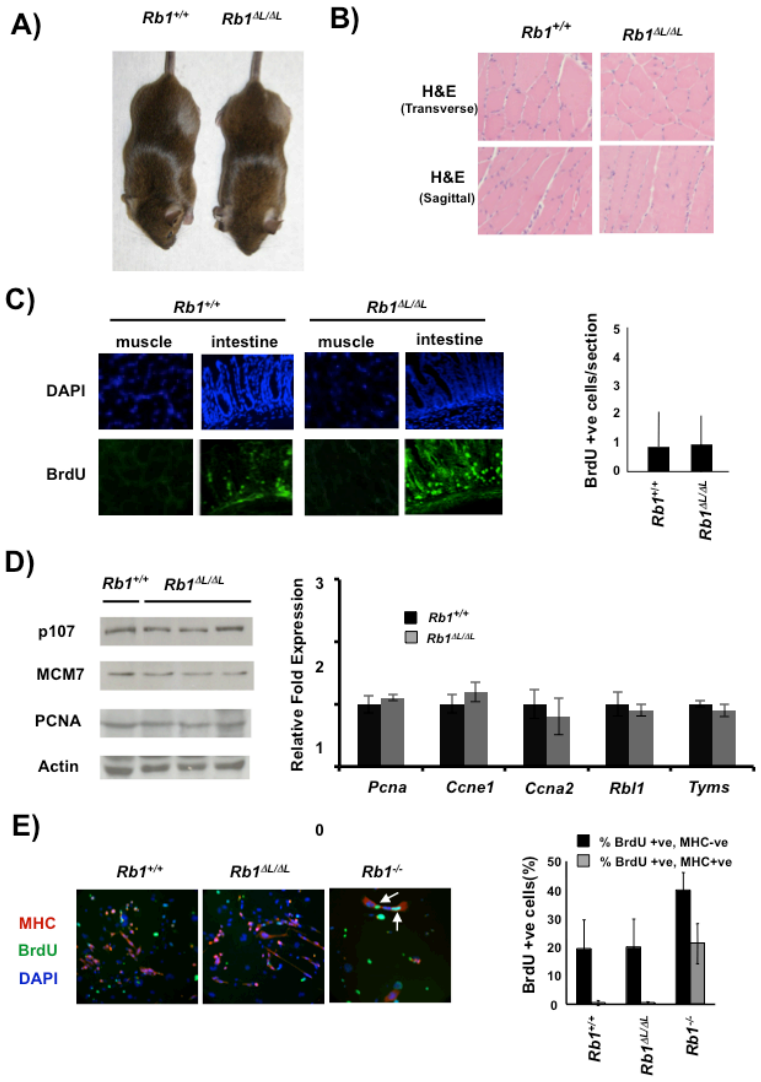

Figure 2.2 Normal cell cycle exit and differentiation of muscle in *Rb1*$^{\Delta L/\Delta L}$ mice

(A) *Rb1*$^{\Delta L/\Delta L}$ mutant animals are viable and appear indistinguishable from wild type littermates. (B) Anterior tibialis muscle tissue from 8 to 10-week old animals was stained with H & E to examine gross morphology of wild type and *Rb1*$^{\Delta L/\Delta L}$ mutants. Transverse and sagittal sections are shown. (C) Cell proliferation in muscle was examined by BrdU staining and the number of BrdU positive cells per microscopic field was quantified and the average displayed in the graph. As a control for detection of BrdU in mature muscle fibers we also stained cryosections of intestinal epithelia prepared from the same mice. (D) mRNA and protein was extracted from the muscle of 6 week old wild type and *Rb1*$^{\Delta L/\Delta L}$ mutant mice. Western blots show expression of known E2F target genes and the graph to the right displays the relative abundance of the specified transcripts. Message levels of acidic ribosomal phospho protein P0 (*Rplp0*) and protein levels of Actin were used as controls. (E) MEFs were infected with MyoD expressing retroviruses and induced to differentiate into myocytes under low serum conditions. Cells were then re-stimulated with 15% FBS and pulse labeled with BrdU (24 hours) to detect DNA synthesis. Myocytes were identified by MHC staining (red), DNA synthesis was detected by BrdU staining (green), and DNA was counterstained with DAPI (blue). The percentage of myocytes (MHC positive) and surrounding fibroblasts (MHC negative) that incorporated BrdU in response to serum is shown in the graph to the right. Error bars in all graphs indicate one standard deviation from the mean of at least three replicates.

To complement the *in situ* analysis of muscle proliferation above, we also analyzed the permanence of cell cycle exit in a cell culture based assay of muscle differentiation. This allows us to directly compare the $Rb1^{\Delta L/\Delta L}$, $Rb1^{-/-}$, and $Rb1^{+/+}$ genotypes, since $Rb1^{-/-}$ myoblasts don't form muscle fibers (Huh et al., 2004a). We infected MEFs with a MyoD expressing retrovirus to induce the formation of myocytes and stimulated differentiation in low serum as described previously (Novitch et al., 1996). Prior reports have revealed that $Rb1^{-/-}$ myocytes generated by this methodology are susceptible to cell cycle re-entry upon serum stimulation (Novitch et al., 1996). The $Rb1^{\Delta L}$ allele readily supports a growth factor resistant cell cycle exit that is indistinguishable from the wild type control (Fig. 2.2E). These data suggest that the $Rb1^{\Delta L}$ mutant is capable of supporting a permanent cell cycle exit in terminal differentiation that is just as robust as wild type. This result stands in stark contrast to the incomplete senescent arrest described in Figure 2.1 because these cell cycle arrest assays start with the same fibroblast cells.

Similarly, it is known that conditional deletion of *Rb1* in the retina causes cell death of ganglions, as well as bipolar and rod cells (Chen et al., 2004a). In addition, *Rb1* deficiency causes differentiation defects in starburst amacrine cells (SACs). Our analysis of $Rb1^{\Delta L/\Delta L}$ mutants reveals that all of these cell types are specified normally, and at the same developmental time as wild type (Fig. 2.3A). Cell types that are unaffected by conditional deletion of pRB are also normal in the $Rb1^{\Delta L/\Delta L}$ mutant (Fig. 2.4), indicating that retinal cells are correctly specified in $Rb1^{\Delta L/\Delta L}$ mice. We also investigated the proliferative status of $Rb1^{\Delta L/\Delta L}$ mutant retinas at both P8 and P18. As shown in Figure 2.3B, proliferation has ceased in $Rb1^{\Delta L/\Delta L}$ retinas at P8 and remains absent at P18. In contrast, proliferation persists in conditional *Rb1* knockouts at both time points as indicated by Ki67 staining (Fig. 2.3B). This reveals that the $Rb1^{\Delta L}$ mutant is capable of mediating normal cell cycle exit during retinal development, further emphasizing that the cell cycle exit and terminal differentiation of long-lived cell types is essentially normal in $Rb1^{\Delta L/\Delta L}$ mice.

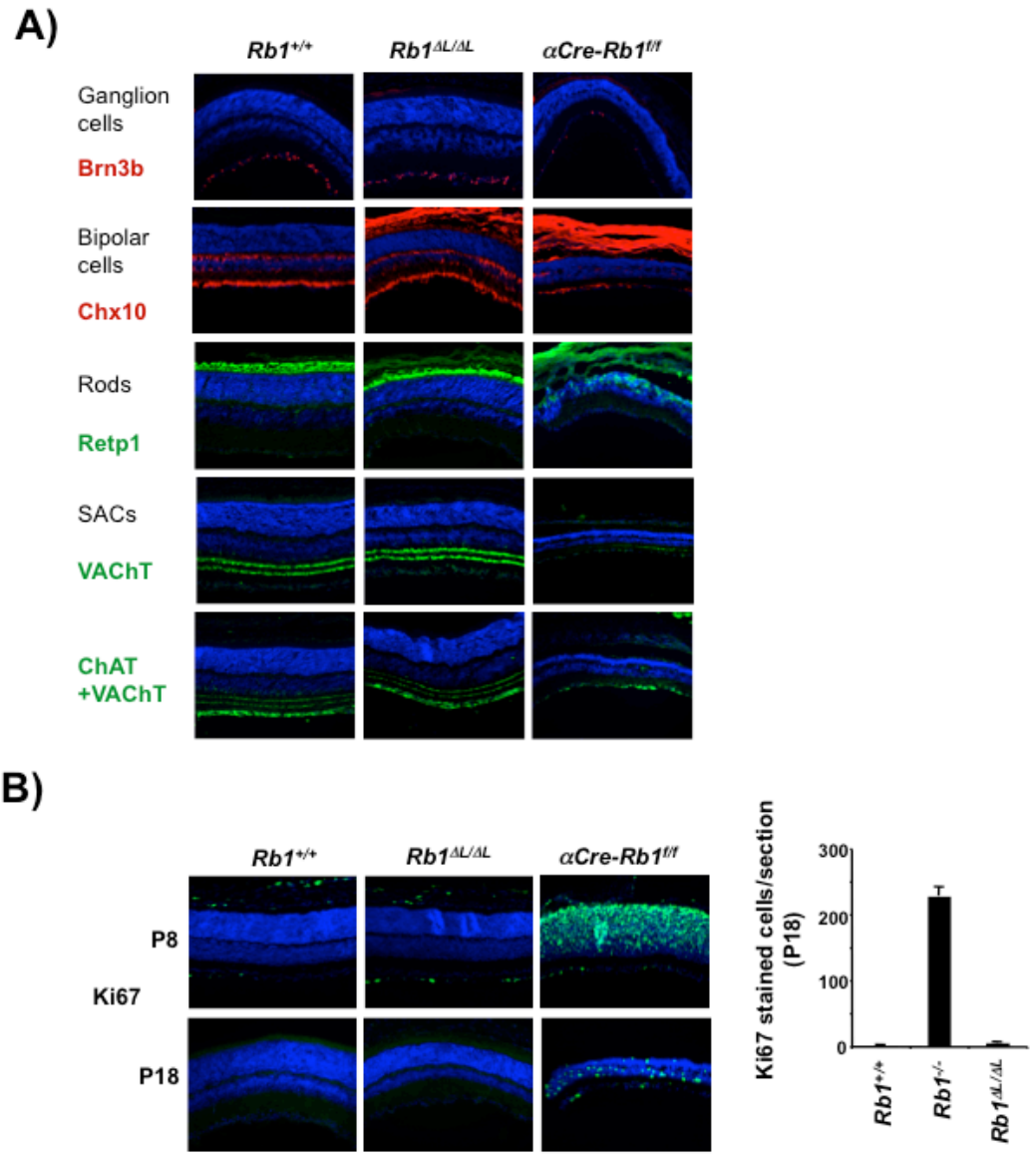

Figure 2.3 Developmental cell cycle arrest is normal in *Rb1*$^{\Delta L/\Delta L}$ mutant retinas

(A) Cross sections of retinas were used to examine morphology and cellular composition in eight day-old newborn mice. Ganglion, bipolar, rod, and starburst amacrine cells (SACs) were stained for the protein marker indicated to the left of each panel (red or green) and nuclei were counterstained with DAPI (blue). α-crystalin-Cre deletion of *Rb1*$^{f/f}$ in the retina and its effects on development in these cells is included as a control. (B) Cell proliferation in differentiated retinal cells was examined by Ki67 staining (green) and counterstained with DAPI (blue) in eight day old and 18 day old mice. α-crystalin-Cre deletion of *Rb1*$^{f/f}$ in the retina and its deregulation of proliferation is included as a control. The number of Ki67 positive cells per tissue section was counted for each genotype and is shown to the right. Error bars in all graphs indicate one standard deviation from the mean of at least three replicates.

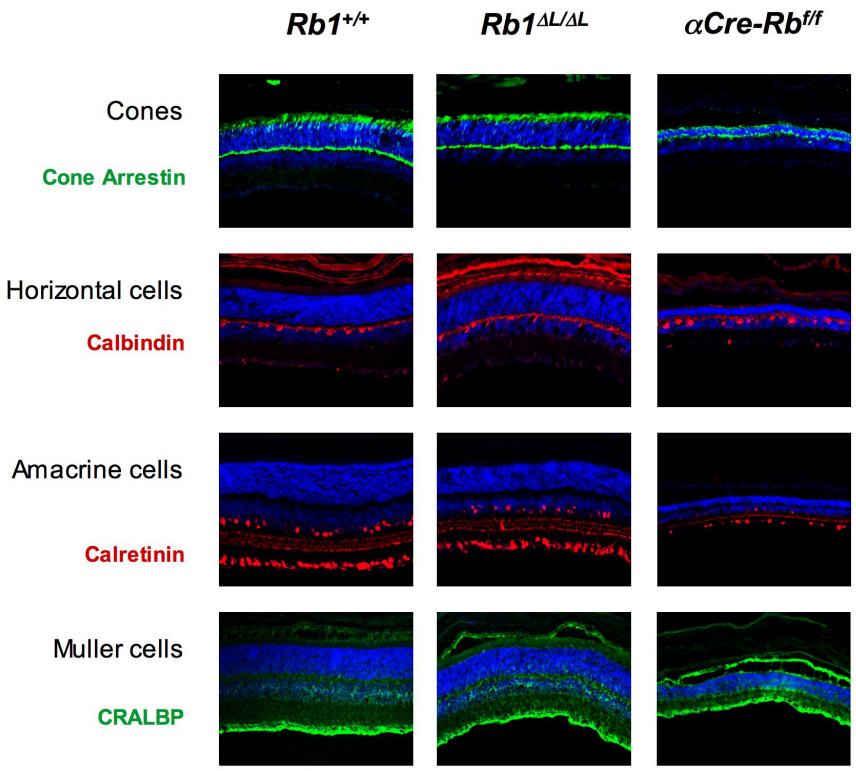

Figure 2.4 Normal specification of cells in the developing *Rb1*$^{\Delta L/\Delta L}$ retina.

P8 retinas were fixed and cryosectioned followed by staining with cell specific retinal markers. Cone cells were stained with cone arrestin, horizontal cells with calbindin, amacrine cells with calretinin, and Muller cells were visualized with CRALBP. Tissues were counterstained with DAPI (blue).

2.3.3 Defective senescence in *Rb1*$^{\Delta L/\Delta L}$ fibroblasts contributes to immortalization

In contrast to the cell cycle exit that occurs normally during development in *Rb1*$^{\Delta L/\Delta L}$ mice, we investigated whether the defective senescent arrest allows cells to escape and resume proliferating. Using a 3T3 culture protocol we passaged wild type and mutant fibroblasts to determine if they have similar proliferative potential by measuring the passage at which they enter senescence. Figure 2.5A shows that they enter senescence at an equivalent passage. DNA replication was measured in successive passages of senescent cultures by BrdU incorporation and levels were found to be elevated in *Rb1*$^{\Delta L/\Delta L}$ mutants (Fig. 2.5B, C). This indicates that *Rb1*$^{\Delta L/\Delta L}$ MEFs respond similarly to a 3T3 culture protocol as they do to other senescence inducing stimuli (Fig. 2.1) that produce an incomplete arrest. To detect early escape from this defective senescence, we continued to culture these fibroblasts and counted the first passage at which they resumed doubling as escape (Fig. 2.5A). Based on this criteria, *Rb1*$^{\Delta L/\Delta L}$ cultures become immortal significantly earlier than wild type controls ($P<0.05$). This suggests that cells from *Rb1*$^{\Delta L/\Delta L}$ mutant mice not only enter into an incomplete senescent state, but this allows them to escape and resume proliferating more readily.

We also sought a developmental comparison for the rapid escape from senescence that we observed in *Rb1*$^{\Delta L/\Delta L}$ 3T3 cells. Unfortunately, none of the experiments characterizing the cell cycle arrest of whole tissues in mutant *Rb1*$^{\Delta L/\Delta L}$ mice in Figures 2.2 or 2.3 are capable of detecting rare cells that undergo sporadic DNA replication. Thus, to search for rare DNA replication events, we analyzed the DNA content of hepatocyte nuclei. While hepatocytes retain proliferative potential for regeneration that separates them from muscle and retinal cells, they become extensively growth arrested in adult mice (Steer, 1996). As mice age, ectopic DNA replication occurs in hepatocytes at a low level, however, many of these cells fail to undergo a subsequent cell division resulting in endoreduplication (Mayhew et al., 2005). Thus, rare replication events that accumulate over time are identifiable by increased nuclear DNA content. Importantly, conditional deletion of pRB in hepatocytes is known to exacerbate this age dependent, endoreduplication effect (Mayhew et al., 2005). Our analysis of *Rb1*$^{\Delta L/\Delta L}$ livers showed

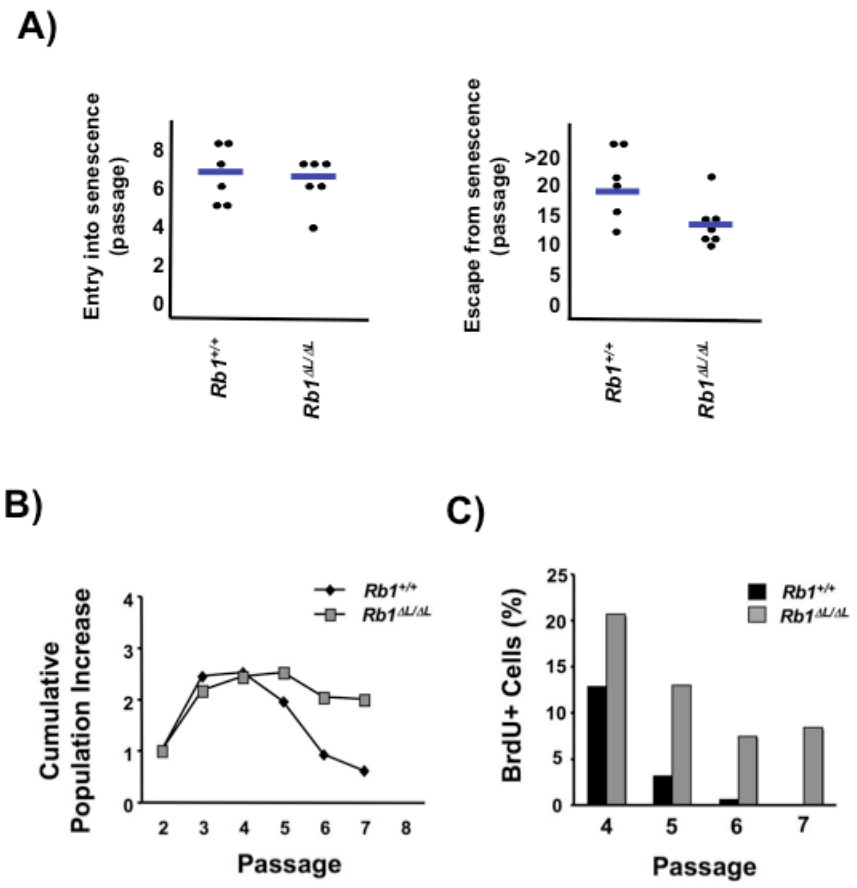

Figure 2.5 Defective senescence of *Rb1*$^{\Delta L/\Delta L}$ mutants contributes to immortalization

(A) MEFs were subjected to a 3T3 culture protocol to induce entry into senescence. We measured the number of passages that it took the cells to senesce and the number of passages it took them to become immortalized. Senescence was defined as the first passage without a population doubling and immortalization was the next passage where cells resumed doubling and continued to double each passage thereafter. Scatter plots showing the passage where each wild type or *Rb1*$^{\Delta L/\Delta L}$ culture ceased to proliferate is shown at left. Plots that reveal when cultures resumed proliferating are shown at right. Horizontal bars represent the mean for each measurement. *P* values are 0.66 (for entry) and 0.04 (escape). (B) Cells were counted at each passage to calculate the cumulative population increase and it is plotted against the passage number. (C) At key passages in this experiment, some cells were grown on cover slips, BrdU labeled, and the percentage of positive cells was determined.

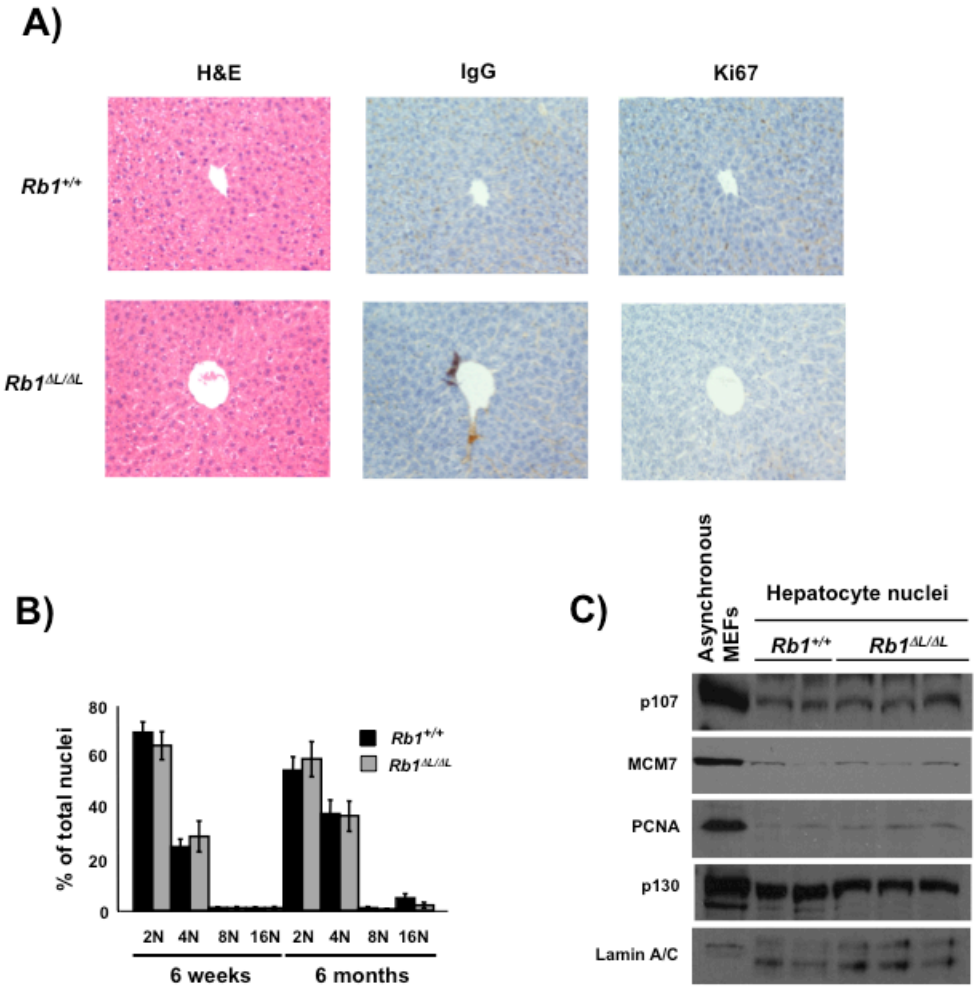

Figure 2.6 Normal long term arrest of hepatocytes in *Rb1*$^{\Delta L/\Delta L}$ mutant

(A) H&E and Immuno-histochemical staining of liver sections from wild type and *Rb1*$^{\Delta L/\Delta L}$ mutants stained with Ki67 antibody (or IgG control). Each field of view is centered on a portal duct to ensure equivalent orientation of the tissue. (B) DNA content of nuclei extracted from livers was analyzed by PI staining and flow cytometry. Each ploidy content category is expressed as a percentage of the total number of nuclei analyzed. Error bars indicate one standard deviation from the mean for at least three replicates. (C) Protein expression of known E2F target genes as well as other pRB family proteins are shown for nuclear extracts prepared from hepatocytes.

that they appear histologically normal by H&E staining of tissue sections (Fig. 2.6A). We also didn't detect any proliferating cells as measured by Ki67 staining in either wild type or mutant liver sections (Fig. 2.6A) suggesting that the growth arrest is normal in $Rb1^{\Delta L/\Delta L}$ livers. We also found that DNA content increases uniformly with age in wild type and $Rb1^{\Delta L/\Delta L}$ animals, indicative of normal control of DNA replication (Fig. 2.6B). In addition, expression levels of E2F targets and other RB family proteins remain normal under these circumstances further suggesting that compensation by related proteins does not underlie the maintenance of cell cycle arrest in $Rb1^{\Delta L/\Delta L}$ hepatocytes (Fig. 2.6C). Because hepatocytes undergo sporadic DNA replication as part of a normal aging process, this analysis shows that even the most sensitive measures of DNA replication support the conclusion that cell cycle exit in development is as robust in $Rb1^{\Delta L/\Delta L}$ mice as wild type controls.

2.3.4 Incomplete senescence in $Rb1^{\Delta L/\Delta L}$ cells is characterized by defective transcriptional repression.

Our initial experiments have revealed that cells from $Rb1^{\Delta L/\Delta L}$ mice are defective in their senescent cell cycle arrest. To explore the cause of this defect further, we investigated the effects of the $Rb1^{\Delta L}$ mutation on transcriptional silencing of E2F target genes. In order to generate an opportunity to manipulate E2F dependent gene transcription in a senescent environment, we induced senescence using oncogenic ras and ectopically expressed human E2F1 by subsequent adenoviral infection. In this way we were able to probe the accessibility and potential for transcriptional activation of E2F responsive promoters. As shown in Figure 2.7A, E2F1 was expressed equally in wild type and mutant cells. In addition, expression levels of three E2F responsive targets, p107, MCM7, and PCNA were increased in the incompletely senescent $Rb1^{\Delta L/\Delta L}$ MEFs and are further elevated by E2F1 expression. More importantly, E2F1 induced higher levels of BrdU incorporation in $Rb1^{\Delta L/\Delta L}$ mutant cells compared to wild type (Fig. 2.7B). Because basal levels of BrdU incorporation in $Rb1^{\Delta L/\Delta L}$ cells are slightly higher than wild type under these conditions (see Fig. 2.1 E), we also calculated the fold induction of BrdU incorporation in response to E2F1 (Fig. 2.7B) and this was also significantly higher in mutant cells ($P<0.05$). Thus, by using ectopic E2F1 expression we have demonstrated

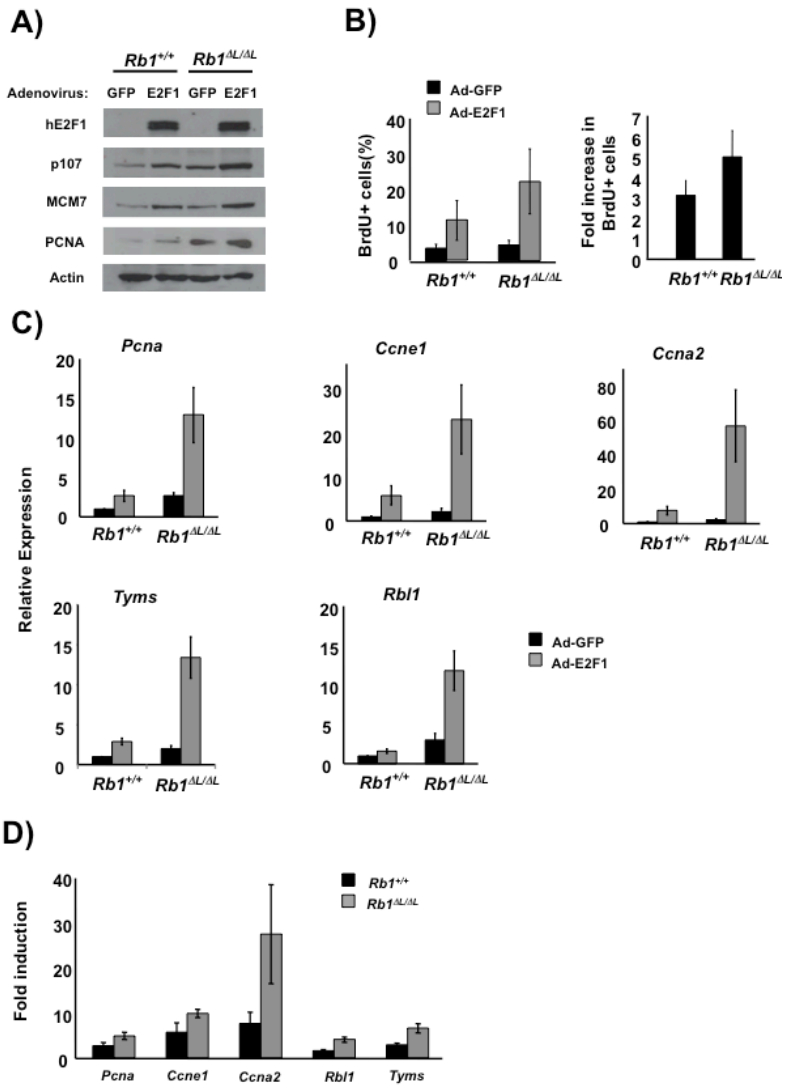

Figure 2.7 Defective repression of E2F target genes in senescent *Rb1*$^{\Delta L/\Delta L}$ MEFs

(A) Ten days following retroviral transduction with oncogenic ras, senescent MEFs were infected with recombinant adenoviruses expressing either GFP or human E2F1 at a multiplicity of infection (MOI) of 100 PFU/cell. The expression level of ectopic E2F1 was measured by western blotting with a human specific α-E2F1 antibody (KH95) after 48hrs. The protein expression level of three known E2F target genes is also shown. Western blotting for Actin serves as a loading control. (B) Synthesis of DNA in response to E2F1 expression was measured by BrdU incorporation. Two days following Ad-E2F1 infection, cells were pulse labeled with BrdU for 16hrs and positive cells were identified by immunofluoresence microscopy. The fold increase in BrdU incorporation between control and E2F1 infected cells was calculated and is shown in the graph on the right. The mean fold increase was compared by a *t*-test (P <0.05). (C) The relative abundance of mRNA corresponding to five E2F target genes is shown. To facilitate comparisons, the expression level in uninfected wild type cells is designated as a relative abundance of one. Expression of acidic ribosomal phospho protein P0 (*Rplp0*) is used as internal control. (D) The fold increase in mRNA abundance in E2F1 expressing *Rb1*$^{\Delta L/\Delta L}$ cells relative to wild-type control is shown for each E2F target gene (P value is <0.05 for each gene). Error bars indicate one standard deviation from the mean for at least three replicates.

that the incomplete senescent cell cycle arrest in *Rb1*$^{\Delta L/\Delta L}$ cells is more susceptible to being overridden by proliferative signals that activate E2F dependent transcription.

To examine the transcriptional effects of E2F1 expression more closely we compared mRNA levels from five well-characterized E2F responsive genes, *Pcna*, *Ccne1*, *Ccna2*, *Rbl1*, and *Tyms* (thymidylate synthase). In each case the expression level was higher in senescent *Rb1*$^{\Delta L/\Delta L}$ cells than in the wild type controls (Fig. 2.7C). Upon E2F1 expression, these target genes were also more readily transcribed as they accumulated to higher levels in the *Rb1*$^{\Delta L/\Delta L}$ mutants. Furthermore, by measuring the fold induction of each E2F target gene, the ability of E2F1 to activate transcription in *Rb1*$^{\Delta L/\Delta L}$ cells was again significantly higher than in wild type (Fig. 2.7D). This demonstrates that cell cycle regulated, E2F-responsive promoters are more readily activated in defectively senescent *Rb1*$^{\Delta L/\Delta L}$ mutants. This suggests that the transcriptional silencing is probably altered in senescent *Rb1*$^{\Delta L/\Delta L}$ cells. Importantly, this difference allows E2F1 expression to stimulate senescent *Rb1*$^{\Delta L/\Delta L}$ cells to synthesize DNA more readily.

2.3.5 *Rb1*$^{\Delta L/\Delta L}$ cells fail to heterochromatinize E2F target gene promoters in senescence.

Given recent studies that have demonstrated the role of chromatin regulation in re-organizing the genome during senescence (Narita et al., 2003b), we wondered if the *Rb1*$^{\Delta L}$ mutation affects this process. We sought to investigate repressive histone tail modifications to determine if they are also altered or absent. In particular, we were interested in H3K9me3 status because one of the histone methyltransferases responsible for adding this modification, Suv39h1, is required for oncogene induced senescence (Braig et al., 2005b) and is reported to interact with pRB through its LXCXE binding cleft (Nielsen et al., 2001).

Chromatin from proliferating and ras induced senescent cells was immunoprecipitated to determine the relative abundance of H3K9me3 at E2F responsive promoters. As a control for our immunoprecipitation experiments we amplified sequences from the imprinted *Airn* promoter. Because of its allele specific expression, we are able to detect H3K9me3 that originates from the silenced allele under all growth

conditions (Regha et al., 2007). In cells that have been induced to senesce with oncogenic ras, H3K9me3 becomes enriched at E2F responsive promoters (Fig. 2.8A). Importantly, H3K9me3 is not enriched at E2F promoters in $Rb1^{\Delta L/\Delta L}$ cells. This analysis reveals that LXCXE interactions by pRB are crucial for assembling heterochromatin in senescence. In addition to the increase in H3K9me3, it has also been proposed that repressive marks like H3K27me3 play an important role in silencing cell cycle genes such as *Ink4a* in an RB family dependent manner (Bracken et al., 2007; Kotake et al., 2007). For these reasons we also investigated H3K27me3 histone tail modifications at the same E2F responsive promoters in senescence (Fig. 2.8B). We also amplified sequences from *HoxD10* homeobox gene promoter that has been shown previously to be enriched for this mark, as an additional control for our immunoprecipitations. This analysis demonstrates that some E2F target genes also increase their abundance of H3K27me3 in senescence when compared with asynchronously growing cells. Interestingly, deposition of this histone tail modification is not dependent on pRB-LXCXE interactions. Given the ability of ectopic E2F1 to activate genes like *Ccne1* in incompletely senescent $Rb1^{\Delta L/\Delta L}$ cells and the fact that only H3K9me3 is added at this promoter in a pRB-LXCXE dependent manner, we suggest that H3K9me3 is a key repressive modification that silences gene expression.

Assembly of repressive heterochromatin has also been implicated in the establishment of a stable cell cycle exit in terminal differentiation (Ait-Si-Ali et al., 2004; Blais et al., 2007; Panteleeva et al., 2007). We next wanted to determine if the epigenetic landscape of the same E2F responsive genes was similar in differentiated muscle and whether it differs between wild type and mutant mice. In agreement with previous work, we found that the H3K9me3 mark can be detected at the promoters of E2F responsive cell cycle genes in muscle (Fig. 2.9 left). We observed that some promoters like *Ccne1 and Mcm3* are enriched for both H3K9me3 and H3K27me3 and in the case of genes like *Mcm5* there is a significant enrichment of H3K27me3 whereas we were unable to detect H3K9me3 levels above background suggesting it has a prominent role in repression of this gene (Fig. 2.9). Surprisingly, neither the deposition of H3K27me3 or H3K9me3 is different between wild type and mutant muscle tissue. This strongly suggests that a different mechanism governs the silencing of E2F responsive genes in terminal

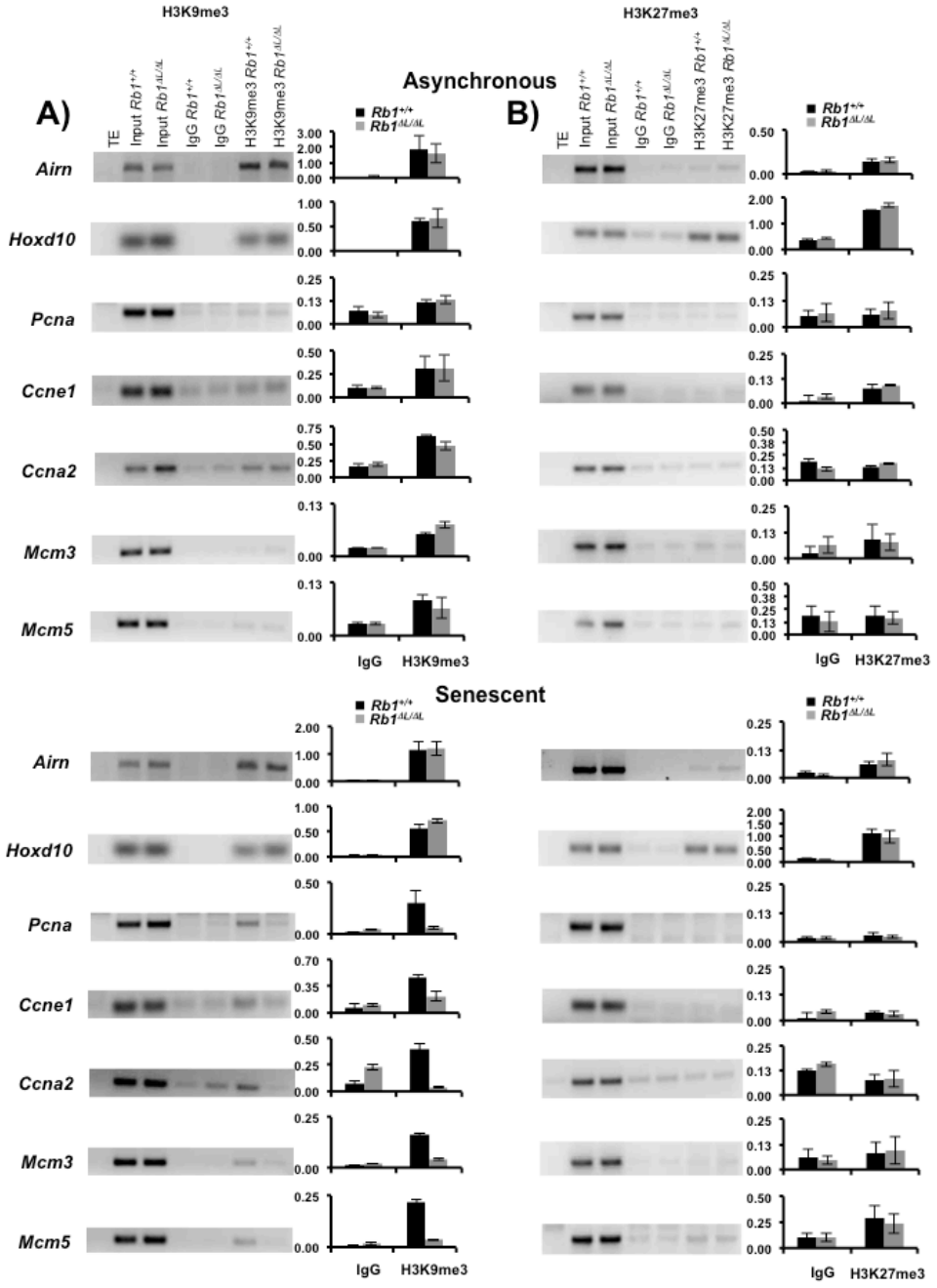

Figure 2.8 Disrupted heterochromatin structure in senescent *Rb1*$^{\Delta L/\Delta L}$ cells

(A and B) Chromatin immunoprecipitations (ChIP) were performed on extracts from asynchronous or ras induced senescent MEFs. Sheared chromatin was precipitated with either rabbit IgG control, anti-H3K9me3, or anti-H3K27me3 antibodies. Input control PCR was performed on 0.5% of chromatin used for each ChIP. Precipitated DNA fragments were amplified by PCR using primers specific for the promoter regions of *Airn*, *Hoxd10*, *Pcna*, *Ccne1*, *Ccna2*, *Mcm3* and *Mcm5*. Band intensities are quantified using image quantification software from Bio-Rad and presented as graphs. Error bars indicate standard deviation from mean value generated from multiple trials of chromatin immunoprecipitations.

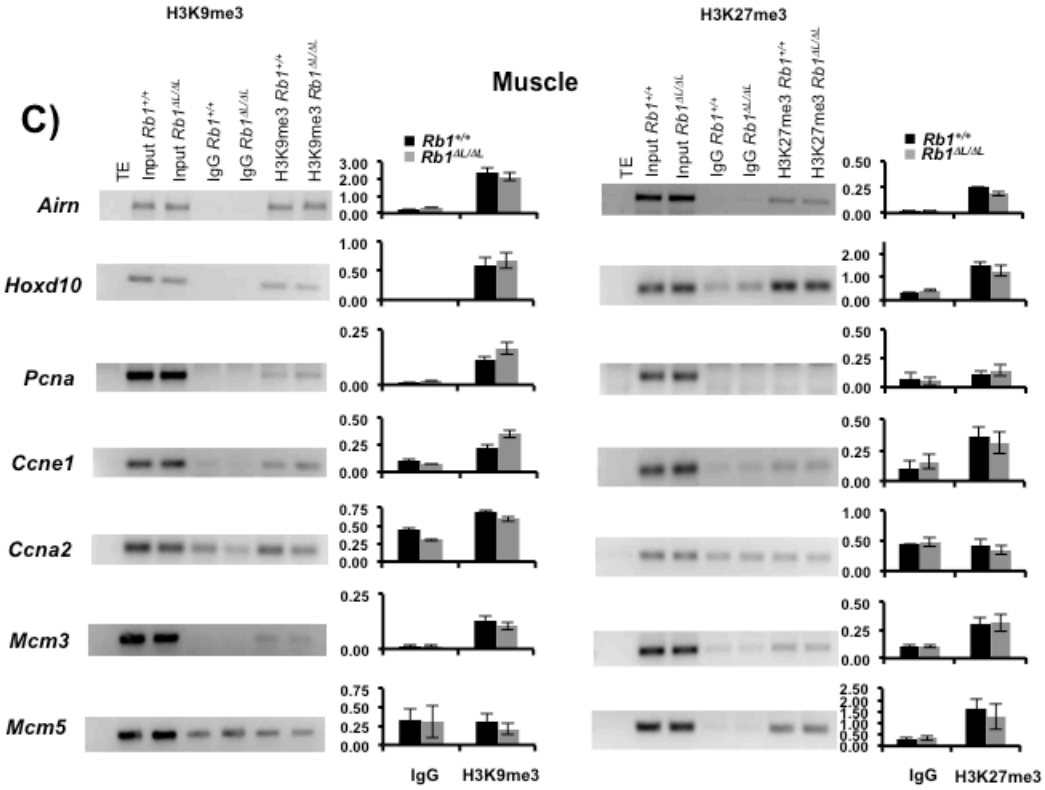

Figure 2.9 Heterochromatin regulation during terminal differentiation is distinct from senescence

Muscle tissue from 6 week old, wild type and mutant mice were used for ChIP with either rabbit IgG control, anti-H3K9me3 (left) or anti-H3K27me3 (right) antibodies as in figure 2.6. Band intensities are quantified same as in fig 2.8.

differentiation. Previous reports demonstrate that H3K9me3 and H3K27me3 deposition at E2F target genes occurs in response to pRB dependent myogenesis (Blais et al., 2007). For these reasons we interpret our results to mean that pRB has multiple growth arrest mechanisms at its disposal, and that the pathways used in cell cycle arrest during senescence and terminal differentiation are fundamentally distinct.

2.4 Discussion

Our work reveals the surprising finding that pRB possesses the ability to block DNA replication in senescence using a fundamentally different mechanism from a permanent cell cycle arrest in development. In particular pRB requires LXCXE type interactions to regulate chromatin structure and silence E2F responsive genes in senescence. This is an important distinction because it demonstrates that pRB uses more than just a single growth suppressive mechanism to block proliferation. It reveals that specific growth arrest signals like DNA damage elicit different functions from pRB than the development programs that govern myogenesis and neurogenesis. It also suggests that different external signals (for example expression of MyoD versus rasV12) could activate different functions of pRB. Although seemingly growth restrictive there lies an important qualitative distinction between these two types of stimuli that activate distinct functions of pRB. Whereas the expression of MyoD in growth restrictive conditions, signal the cells to exit the cell cycle and differentiate, expression of oncogenic ras under normal growth conditions activates conflicting signals by driving rapid proliferation on one hand and activating growth arrest signals by inducing DNA damage on the other. We think it is in such a context of persistent conflicting signals that specific functions of pRB are activated, further highlighting its role as a tumor suppressor.

This study emphasizes that cell cycle arrest in senescence requires a repressor module containing E2F-pRB and a chromatin regulatory component (Fig. 2.10). Rowland *et al.* have previously shown that expression of a pRB binding deficient mutant of E2F3 can disrupt pRB-E2F function in senescence, demonstrating the need for E2F to recruit this complex to promoters (Rowland et al., 2002). The response of $Rb1^{\Delta L/\Delta L}$ cells

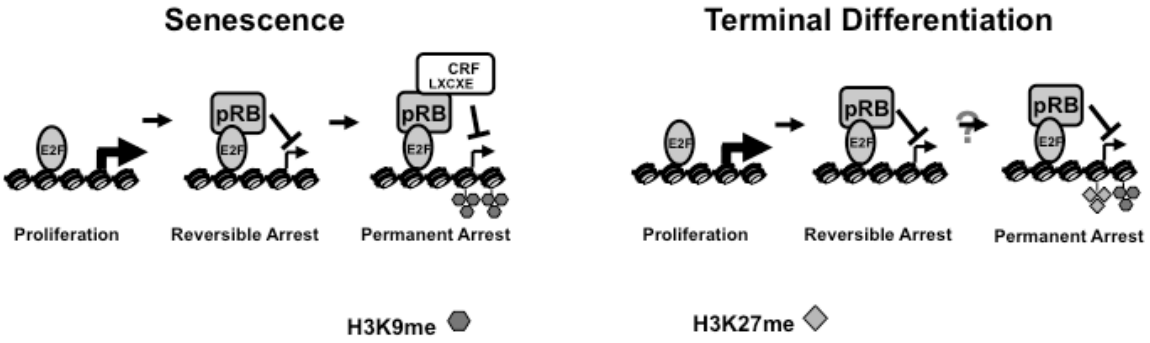

Figure 2.10 Model of cell cycle exit regulation in senescence and terminal differentiation

During the induction of a senescent arrest, pRB-E2F interactions regulate proliferation sufficiently to induce a reversible arrest state. Incomplete senescence of *Rb1*$^{\Delta L/\Delta L}$ cells appears to reach this state where they remain susceptible to re-replication of their DNA. Establishment of a heterochromatin barrier that can block inappropriate cell cycle re-entry is dependent on pRB-LXCXE interactions and H3K9me3 histone tail modifications. Cell cycle exit associated with terminal differentiation requires pRB regulation of E2Fs. Through unknown mechanisms the initial withdrawal from the cell cycle becomes permanent. E2F target promoters become heterochromatinized with H3K27me3 and H3K9me3 modifications in a manner that is independent of pRB-LXCXE interactions.

to DNA damage indicates that the initial steps in cell cycle arrest take place normally allowing these cells to reach a reversible arrest. This suggests that pRB-E2F interactions are sufficient to mediate this initial step (Fig. 2.10). The low level of DNA synthesis that persists over time in $Rb1^{\Delta L/\Delta L}$ mutants suggests that the true role for chromatin regulation by pRB in senescence is to function as a failsafe mechanism in cell cycle arrest that establishes permanence. Because complete cell cycle exit in senescence is dependent on chromatin remodeling, we describe pRB's role at this step as a checkpoint.

The discovery that pRB-LXCXE interactions are dispensable for a terminal differentiation related cell cycle arrest is very surprising. As stated earlier, an E2F-pRB-chromatin regulatory complex such as that shown for a permanent arrest in Figure 2.10 (left) is highlighted in many reviews of pRB function as controlling cell cycle exit in a ubiquitous arrest scenario that includes terminal differentiation (Brehm and Kouzarides, 1999; Burkhart and Sage, 2008; Classon and Harlow, 2002b; Harbour and Dean, 2000; Korenjak and Brehm, 2005). We offer the following explanations as well as our own data in support of the model of terminal differentiation shown in Figure 2.10 (right) where LXCXE dependent chromatin regulation is dispensable. We think that pRB's role in a developmentally induced cell cycle exit may be accomplished largely through negative regulation of activator E2F activity. This interpretation is supported by the fact that a number of differentiation defects caused by complete loss of pRB can be rescued by crossing to null alleles of activator E2Fs. In the murine retina, it is known that conditional deletion of *Rb1* triggers ectopic division and death of ganglions, bipolar, and rod cells (Chen et al., 2004a). These defects in terminal differentiation are reversed by *E2f1* deficiency. Furthermore, ablation of *Rb1* in the telencephalon has been reported to dissociate proliferative control from the initiation of neuronal differentiation (Ferguson et al., 2002). Ectopic cell division in the intermediate zone and cortical plate regions of $Rb1^{-/-}$ brain tissue in these mice can be suppressed by *E2f1* or *E2f3* deficiency (McClellan et al., 2007). Beyond E2F regulation, a number of reports have also shown pRB dependent effects on chromatin in terminal differentiation of muscle that may, on the surface, seem to contradict our model. Ablation of *Rb1* in skeletal muscle progenitors has been demonstrated to lead to complete failure of myogenesis (Huh et al., 2004a), and recent experiments using RNAi to deplete pRB expression in myotubes indicates that cell

cycle re-entry is triggered in its absence (Blais et al., 2007). For these reasons formation of myotubes and resulting chromatin changes are clearly pRB dependent. However, pRB is also able to influence the activity of differentiation inducing factors like ID2 and MyoD and through molecules like these it may regulate chromatin in differentiation indirectly (Burkhart and Sage, 2008). For these reasons we suggest that chromatin regulation in terminal differentiation of muscle that is pRB-dependent is either an indirect consequence of cell cycle exit, is independent of LXCXE interactions with pRB, or is induced indirectly through pro-differentiation factors (Fig. 2.10, right).

In addition to senescence, we have also determined that pRB-LXCXE interactions are critical to TGF-β regulation of continuously proliferating mammary epithelial cells (Francis et al., 2009a). While this is a different growth regulatory paradigm, the ability of TGF-β to induce senescence through chronic stimulation further suggests that pRB-LXCXE interactions can be implicated in a broad, stress responsive growth control program (Lin et al., 2004). It is tempting to speculate that the pRB-LXCXE dependent arrest pathway plays a key role in pRB's tumor suppressor function. We have not detected spontaneous tumors in our $Rb1^{\Delta L/\Delta L}$ mutants (Coschi et al., 2010). However, it is noteworthy that the $Rb1^{\Delta L}$ mutation doesn't abrogate senescence completely, but uncouples its permanence from the initial arrest. Other genetically modified strains of mice whose lesions completely abrogate this senescence arrest pathway, such as $Ink4a^{-/-}$ mice, already have surprisingly low rates of spontaneous tumorigenesis themselves (Krimpenfort et al., 2001; Sharpless et al., 2001). Future work to determine the importance of heterochromatin at E2F responsive targets in senescence will require crosses to transgenic mice with defined oncogenic lesions. In this way we will be able to directly relate the chromatin assembly step in senescence to cancer progression.

Intriguingly, our data reveal an unexpected parallel between pRB and p53 in mammalian physiology. Like our $Rb1^{\Delta L/\Delta L}$ mice, $Trp53^{-/-}$ mice are relatively normal developmentally (Donehower et al., 1992). While, p53's role in responding to cancer causing insults like DNA damage is well known, only recently has it been demonstrated that p53's role in stellate cell senescence is essential for the liver to respond appropriately to chemical toxicity and avoid fibrosis (Krizhanovsky et al., 2008). The unique role for

pRB-LXCXE regulation of chromatin in senescence that we describe offers a similar glimpse at a fundamental stress response mechanism. Indeed, other reports have suggested a role for pRB in stress responses (MacLeod, 2008; Mason-Richie et al., 2008). In particular, lung epithelium appears to use pRB in a very specific role in controlling proliferation following injury, but not in development (Mason-Richie et al., 2008). Thus, it seems that pRB plays a unique role in this growth control paradigm that developed to respond to stressful exogenous stimuli, including DNA damage, the release of TGF-β in response to tissue trauma, or as a protective response to chemical toxicity. Such responses, which are largely independent of cell cycle control during development, imply that a stress-responsive growth control program is a pervasive and important aspect of mammalian physiology. It is difficult to know to what degree evolution has selected for anti-cancer functions in the genes that code for p53 and pRB, however, the involvement of these master regulators in a stress specific growth arrest reveals an important biological feature of proliferative control. Genes involved in a checkpoint that is stress responsive, as opposed to ones that are largely regulated by developmental cues, may offer a starting point for growth control mechanisms that in present day offer anti-oncogenic properties as genetic damage accumulates in response to environmental pressures.

2.5 Materials and Methods

2.5.1 Mice

The generation of $Rb1^{\Delta L/\Delta L}$ mutant mice has been described previously (Isaac et al., 2006b). $Rb1^{-/-}$ mice were obtained from MMHCC. Mice bearing $Rb1^{f/f}$ alleles and the α-crystalin-Cre transgene were generated as before (Chen et al., 2004a). All animals were housed and handled according to Canadian Council on Animal Care regulations.

2.5.2 Cell culture

Mouse embryonic fibroblasts (MEFs) were generated from d13.5 embryos using standard procedures and cultured as previously described (Isaac et al., 2006b). Retroviral transduction with pBABE-HrasV12 was as reported by Serrano et al. (Serrano et al., 1997b) and viruses were packaged in Bosc-23 cells. Cells infected with viruses encoding

ras were selected in 4 μg/ml puromycin for at least 3 days before processing for further experiments using flow cytometry, microscopy, or extract preparation. Senescent cells prepared by this method were allowed to senescence for at least 10 days following retroviral infection. Cells induced to senesce with γ-irradiation were exposed to 15 Gys. Senescence associated β-galactosidase (SA-β-Gal) staining was performed as described (Serrano et al., 1997b). Infections with Ad-E2F1 were according to standard methods and cells were cultured for an additional 48 hours before labeling with BrdU for 16 hours, or preparing extracts. Myogenic differentiation was carried out by infecting MEFs with a pBABE-MyoD based retrovirus and following the differentiation protocol of Novitch (Novitch et al., 1996). Cells were re-stimulated with 15% serum and labeled with BrdU for 24 hours. 3T3 culture assays were carried out following previously reported methods (Todaro and Green, 1963), as modified by Classon et al. (Classon et al., 2000).

2.5.3 Histology and Fluorescence Microscopy

Haematoxylin and Eosin (H & E) stained tissues were fixed in formalin, embedded and stained with H&E using standard procedures. All other tissues were fixed in optimum cutting temperature (OCT) compound and embedded for cryosectioning. Staining of retinal sections was carried out as described by Chen (Chen et al., 2004a), and anti-BrdU staining was as recommended by the manufacturer (Becton-Dickinson, San Jose, California). Cell cultures were fixed and permeabilized in alcohol, blocked, and stained for BrdU or protein markers as previously described (Isaac et al., 2006b). Antibodies against MHC were obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

2.5.4 Quantitation of DNA, protein, and mRNA

DNA content and BrdU incorporation were measured by flow cytometry in Fig. 2.1A and C as described in Isaac et al. (Isaac et al., 2006b). All other measurements of BrdU incorporation were generated from *in situ* staining and microscopic evaluation described above. Flow cytometry measurements of hepatocyte nuclear DNA content were as described by Mayhew et al. (Mayhew et al., 2005). Protein expression levels were detected by western blotting using antibodies against E2F1 (KH95), p107 (C-18),

PCNA (pc10), p130 (C-20) and MCM7 (141.2) from Santa Cruz. Actin (Sigma, A2066) or Lamin A/C (Chemicon, MAB3211) levels were detected as loading controls. Message levels for *Pcna*, *Ccne1*, *Ccna2*, *Tyms*, and *Rbl1* were detected using the Quantigene Plex 2.0 reagent system from Panomics (Fremont, CA) and quantified by comparison with the message for acidic ribosomal phosphoprotein P0 (*Rplp0*) using a BioPlex200 multiplex analysis system according to Panomics instructions. Chromatin immunoprecipitation assays were performed as described previously using anti-H3K9me3 and H3K27me3 antibodies (Upstate) and 2 x 10^7 cells per immunoprecipitation (Aparicio et al., 2005). DNA released from precipitated complexes was amplified by PCR using primers specific to the promoter regions of *Airn* (AGG GTG AAA AGC TGC ACA AG and CCC TGA TCA CAG AAC CCT TC) (Regha et al., 2007), *Pcna* (CTG CGC GAG GTC ATG ACG CCA and CTT CCG TGG CGC GGA AAC TTC C), *Ccne1* (TGA GGG GCT CGC AGC CCT CG and CCC GGC TTC GAG CGG GAC AT), *Mcm3* (GAA TGC AGT GCT TCC TAG CC and CGG AAG TTT ATG GTG GAG GA) (Blais et al., 2007), *Mcm5* (AAC CAA TAG GAG CGC AGA GA and AAG CCC GAC ATG ACT GTA CC) (Blais et al., 2007). *HoxD10* (GCT GAA AAC CTC CCC ATC TT and CCT ACT TGG CGC ATT TTC TC), and *Ccna2* (ATC CAC TGA GCA GCA GAG AT and TTG TAG TTC AAG TAG CCC GCG). All primer sequences are oriented in 5`-3` direction.

2.6 References

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Chapter 3

3 The Retinoblastoma protein and PML collaborate to organize heterochromatin and silence E2F responsive genes during senescence

3.1 Abstract

Cellular senescence is characterized by silencing of genes involved in DNA replication and cell cycle progression. Stable repression is crucial for preventing inappropriate DNA synthesis and maintaining a prolonged senescent state. Many of these genes are targets for E2F transcription factors. The pRB pathway plays a major role in senescence by directly repressing E2Fs and also by regulating chromatin at the promoters of E2F target genes using its LXCXE cleft dependent interactions. In this study, we sought to investigate the mechanisms by which pRB stably silences E2F target gene transcription during cellular senescence. We report that in mouse embryonic fibroblasts, endogenous Promyelocytic leukemia protein (PML) associates with E2F target genes in a pRB LXCXE dependent manner during HrasV12 induced senescence. Furthermore, using a PML-IV induced senescence model, we show that the pRB LXCXE binding cleft is essential for PML association with gene promoters, heterochromatinization and silencing of E2F target genes. GST pull down assays show that pRB can interact with PML specifically during senescence, implicating an actively regulated assembly step that brings PML and pRB together to establish heterochromatin and create a permanent cell cycle arrest.

3.2 Introduction

Cellular senescence is a stable cell cycle exit that protects cells from transformation and prevents malignancy (Campisi, 2001). Cellular senescence is characterized by cell cycle arrest, repression of proliferative genes, and activation of growth suppressing genes (Chicas et al., 2010). Senescence inducing stimuli such as telomere shortening, expression of activated oncogenes, and DNA damaging agents cause DNA damage and subsequent activation of the DNA damage response (Bartkova et al.,

2006; Di Micco et al., 2006; Mallette and Ferbeyre, 2007). This engages key tumor suppressor pathways regulated by p53 and pRB proteins that act as the effectors of senescence by inducing cell cycle arrest (Campisi, 2001).

One of the defining characteristics of senescence is its permanence and senescent cells are highly refractory to growth promoting signals (Campisi, 2005). Maintaining this stable cell cycle arrest is also considered to be critical to the tumor suppressive role of senescence *in vivo* (Braig et al., 2005). Key to the maintenance of a permanent cell cycle arrest is stable repression of proliferative genes involved in DNA replication and cell cycle progression (Chicas et al., 2010). Senescence is often associated with heterochromatin assembly and this is thought to contribute to stable gene silencing (Narita et al., 2003). In general, there is enrichment of transcriptionally repressive histone modifications such as H3K9me3 and H3K27me3 and a decrease in activating marks such as H3K4me3 on proliferative gene promoters (Chandra et al., 2012; Chicas et al., 2012; Talluri et al., 2010). Furthermore, in some cell types such as IMR90 fibroblasts these chromatin changes are accompanied by pronounced compaction of whole chromosomes into structures that are called SAHFs (senescence associated heterochromatic foci) (Funayama et al., 2006; Narita et al., 2003; Zhang et al., 2007). Although the precise contribution of each of these chromatin changes to the senescent state is not fully understood, they are proposed to contribute to the permanence of senescent arrest by stably silencing proliferative genes and preventing cell cycle entry (Beausejour et al., 2003). There is some *in vivo* evidence supporting this, as mice lacking the enzymes responsible for these repressive histone modifications show defective chromatin assembly and increased susceptibility to cancer, suggesting that chromatin changes contribute to the tumor suppressive role of senescence (Braig et al., 2005).

The pRB-E2F pathway is a key tumor suppressor pathway that regulates the expression of a number of genes involved in DNA synthesis and cell cycle advancement in response to growth factor stimuli (Dimova and Dyson, 2005). Many of these proliferative genes are direct targets of E2F transcription factors, which in turn are negatively regulated by pRB family proteins. This places the pRB-E2F pathway at the core of the cellular senescence response. Genetic models have confirmed this hypothesis

where in MEFs lacking all the RB family proteins (TKO MEFs) fail to senesce, and immortalize spontaneously in culture (Dannenberg et al., 2000; Sage et al., 2000). Amongst the pocket protein family, pRB has a unique role in senescence. pRB is required for repression of key cell cycle genes and prevents DNA synthesis in response to oncogene expression (Chicas et al., 2010). Furthermore, acute knock down of pRB alone is sufficient to induce DNA synthesis and cell cycle re-entry in senescent MEFs, suggesting a crucial role for pRB in the maintenance of a stable senescent state (Sage et al., 2003). This unique role for pRB can be attributed, at least in part, to its ability to regulate the heterochromatinization of cell cycle gene promoters and stable silencing of these genes (Talluri et al., 2010). However, the mechanistic role of pRB in establishing stable senescence is not understood. Taken together, heterochromatin changes accompany senescence induced cell cycle arrest, however, it is unclear if these are a direct effect of pRB, or an indirect consequence of its other functions.

The Pro-myelocytic leukemia protein (PML) is essential for senescence (Bischof et al., 2002; Ferbeyre et al., 2000; Pearson et al., 2000). PML is the primary component of PML nuclear bodies, the sub nuclear structures that increase in abundance in response to a variety of cellular stresses. Expression of oncogenic HrasV12 in fibroblasts results in a dramatic increase in the number and size of PML nuclear bodies (Ferbeyre et al., 2000). The essential role for PML in senescence comes from the observation that fibroblasts from *Pml*$^{-/-}$ embryos fail to senesce and continue proliferating in response to HrasV12 (Bischof et al., 2002). Furthermore, forced expression of PML is sufficient to induce senescence in primary fibroblasts. The *Pml* gene is subject to extensive alternate splicing resulting in at least 7 major isoforms PML I-VII that differ mainly in their C-terminal region (Jensen et al., 2001). PML-IV, among the major isoforms, is the only one able to induce senescence when overexpressed, suggesting an important role for this isoform (Bischof et al., 2002). However, PML-IV fails to induce senescence when expressed in *Pml*$^{-/-}$ MEFs suggesting that other isoforms are also required for efficient induction of senescence (Bischof et al., 2002).

The precise role of PML and its constituent nuclear bodies during senescence is an area of intense research. A functional co-operation between PML and pRB-E2F

pathways during senescence was recently reported (Vernier et al., 2011). pRB and E2Fs were shown to localize to the PML nuclear bodies during senescence and disruption of pRB-E2F interactions, or degradation of RB family proteins by expression of human papilloma virus E7, was sufficient to compromise PML-IV induced senescence. This association between PML and pRB-E2F is proposed to be responsible for repression of E2Fs and their target gene expression. However, since HPV-E7 inhibits RB family proteins and PML alike, the precise aspects of pRB or PML function that are required for senescence remain unknown.

We previously showed that MEFs from a gene-targeted mouse carrying a mutant pRB that is specifically defective for LXCXE type interactions (called $Rb1^{\Delta L}$) are defective for stable repression of E2F target genes during oncogene-induced senescence (Talluri et al., 2010). This mutation also compromises the stability of senescence arrest and enables escape. In the current study, we explored the mechanism of pRB mediated silencing and heterochromatinization of E2F responsive genes using two different senescence contexts, oncogene induced senescence (HrasV12) and PML induced senescence (PML-IV). Here we show that endogenous PML is enriched at the promoters of E2F target genes in a pRB-LXCXE dependent manner during both forms of senescence. The same E2F target genes fail to be repressed in $Rb1^{\Delta L/\Delta L}$ MEFs overexpressing either HrasV12 or PML-IV. Interestingly, $Rb1^{\Delta L/\Delta L}$ MEFs overexpressing PML-IV fail to enrich the repressive histone mark H3K9me3 at *Ccne1* and *Mcm3* gene promoters. This suggests a requirement for PML recruitment by pRB-LXCXE type interactions to induce heterochromatinization and gene silencing. Furthermore, using GST pull down experiments we show that PML is only capable of binding pRB under senescent growth conditions and these interactions are disrupted by mutations in the pRB-LXCXE binding cleft. Our experiments support a model in which pRB interacts with PML in a LXCXE cleft dependent manner and this complex mediates heterochromatinization and silencing of the E2F genes during senescence. Taken together with previous reports in the literature, our data demonstrates that the interaction between pRB and PML is critical to switching from a transient arrest state to a permanent one.

3.3 Results

3.3.1 Defective enrichment of PML on E2F target gene promoters in *Rb1*$^{\Delta L/\Delta L}$ cells during senescence

In a previous study we investigated the role of pRB-LXCXE interactions in cellular senescence using MEFs derived from *Rb1*$^{\Delta L/\Delta L}$ mice (Talluri et al., 2010). We reported that *Rb1*$^{\Delta L/\Delta L}$ MEFs enter a state of partial senescence in response to oncogenic HrasV12 expression in which they take on many of the morphological features of senescent cells, but fail to stably repress E2F target genes, and these genes remain susceptible to activation by ectopic stimuli. Importantly, the *Rb1*$^{\Delta L}$ mutation allows partially senescent *Rb1*$^{\Delta L/\Delta L}$ MEFs to initiate DNA synthesis, re-enter the cell cycle and resume proliferation. We found defective enrichment of the repressive histone modification H3K9me3 on E2F target gene promoters in *Rb1*$^{\Delta L/\Delta L}$ MEFs during senescence ((Talluri et al., 2010) and Fig. 3.1A). Our goal in this study was to use *Rb1*$^{\Delta L/\Delta L}$ cells to identify components of the switch mechanism that converts reversible growth arrest into permanent withdrawal from the cell cycle through heterochromatin formation at E2F promoters.

We searched for proteins whose association with E2F responsive promoters in chromatin immunoprecipitation assays (ChIP) is sensitive to the *Rb1*$^{\Delta L}$ mutation. We examined the *Ccne1* and *Mcm3* gene promoters as these genes are key targets of pRB in proliferative control during senescence (Chicas et al., 2010). As shown in Fig. 3.1B endogenous PML is enriched at both these promoters during senescence in *Rb1*$^{+/+}$ cells. Strikingly, this enrichment is eliminated in *Rb1*$^{\Delta L/\Delta L}$ cells suggesting that PML requires pRB-LXCXE binding cleft mediated interactions for recruitment. Furthermore, pRB is equally enriched at these promoters in both *Rb1*$^{+/+}$ & *Rb1*$^{\Delta L/\Delta L}$ cells (Fig. 3.1C). This shows that while pRB$^{\Delta L}$ is capable of binding to E2F target genes during senescence this mutation specifically disrupts PML association with these promoters, suggesting that it might participate in the switch from short term to long term growth arrest.

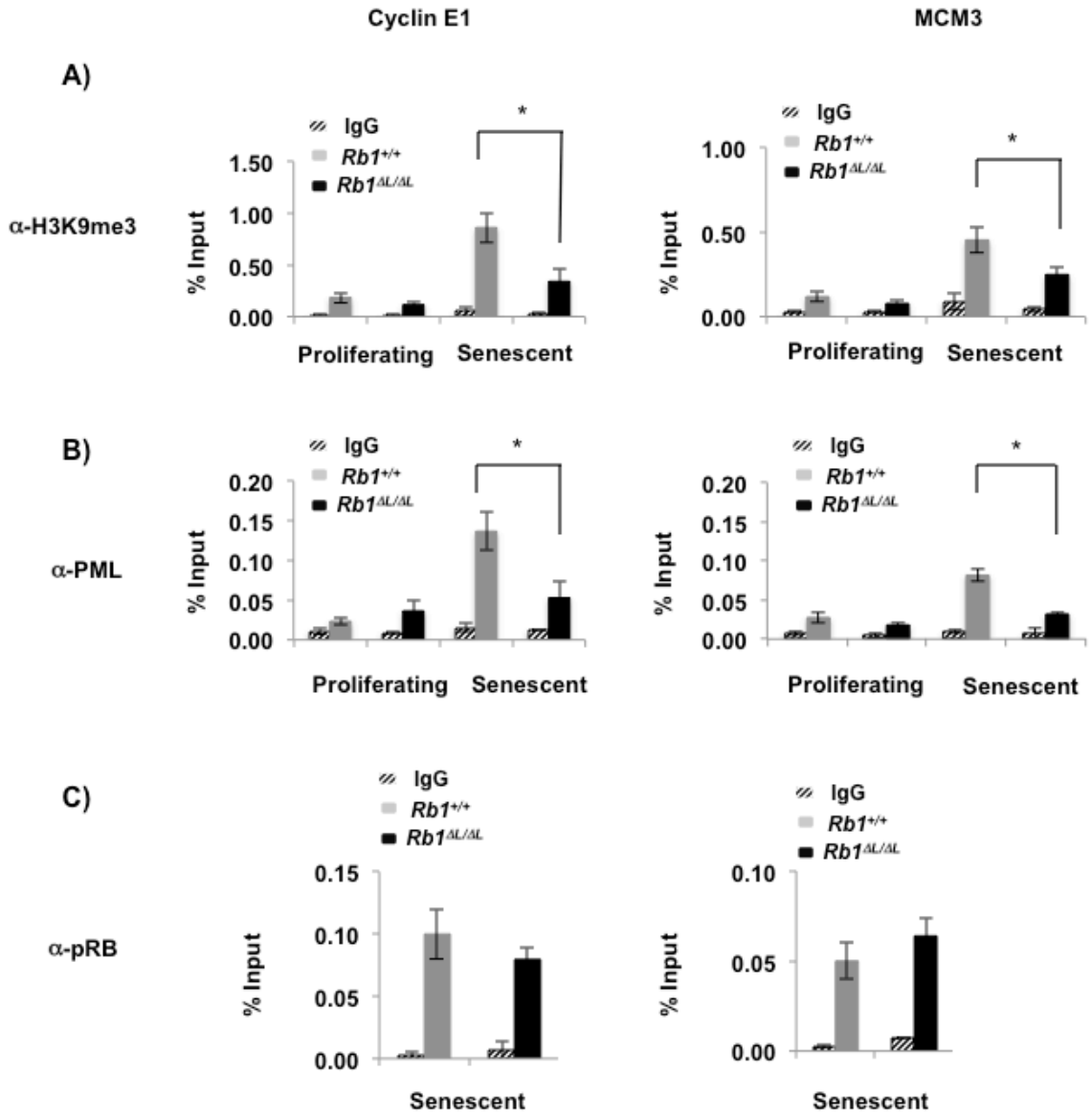

Figure 3.1 Defective enrichment of the PML (Pro-myelocytic leukemia) protein on E2F responsive cell cycle gene promoters in senescent *Rb1*$^{\Delta L/\Delta L}$ cells

Asynchronously growing wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs were induced to senesce by retroviral-mediated expression of oncogenic HrasV12. Chromatin from proliferating and senescent cells was used for chromatin immunoprecipitation. Real time PCR was used to amplify the immunoprecipitated DNA using primers specific to Cyclin E1 (left) and Mcm3 (right). The quantity of precipitated DNA is represented as percent of input chromatin. **(A)** ChIP of proliferating and senescent cells of the indicated genotypes using a α-H3K9me3 antibody or an IgG control. **(B)** ChIP of chromatin from proliferating and senescent wild type and *Rb1*$^{\Delta L/\Delta L}$ cells using a α-PML antibody and an IgG control. **(C)** ChIP on senescent wild type and *Rb1*$^{\Delta L/\Delta L}$ cells using a α-pRB antibody and an IgG control. Error bars indicate one standard deviation from the mean, n = 3. An asterisk indicates a statistically significant difference (*t*-test, $P<0.05$).

3.3.2 Early events during senescence induction occur normally in *Rb1*$^{\Delta L/\Delta L}$ MEFs

Expression of oncogenic ras in primary fibroblasts induces hyper proliferation resulting in replicative stress and DNA damage (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). This leads to the activation of DNA damage signaling and activation of p53 and pRB pathways. Oncogenic ras expression also leads to induction of PML and PML nuclear body formation in a p53 dependent manner (Ferbeyre et al., 2000). Persistent activation of the DNA damage response has also been shown to be important for the maintenance of senescence arrest (Di Micco et al., 2006). We wanted to investigate whether these signaling events that are required for senescence induction are intact in *Rb1*$^{\Delta L/\Delta L}$ MEFs and could explain failure to recruit PML to E2F regulated promoters in senescent *Rb1*$^{\Delta L/\Delta L}$ cells.

First we tested if DNA damage signaling is intact in *Rb1*$^{\Delta L/\Delta L}$ MEFs and if it is activated in response to ras similar to wild type controls. *Rb1*$^{+/+}$ & *Rb1*$^{\Delta L/\Delta L}$ MEFs induced to senesce by expression of oncogenic HrasV12 were stained with antibodies against γH2AX a marker of DNA double strand breaks. As a control, we also assessed DNA damage in low passage, proliferating MEFs. As shown in (Fig. 3.2A and B) HrasV12 expression induces a significant increase in the number of γH2AX foci both in wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs. The damage foci can be seen very early after the expression of HrasV12 and this damage also persists during senescence in both genotypes.

We next determined if PML bodies are formed normally in our mutant background. We used immunofluorescence staining with a α-PML antibody in *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MEFs expressing oncogenic HrasV12. We observed a clear increase in the number of PML nuclear bodies in senescent cells compared to asynchronously proliferating MEFs (Fig. 3.2C). Importantly, *Rb1*$^{\Delta L/\Delta L}$ MEFs showed accumulation of PML bodies similar to wild type cells (Fig. 3.2C and 3.2D). We observed a significant shift towards more PML bodies per nucleus (>10) in MEFs induced to senesce by HrasV12 expression (Fig. 3.2D).

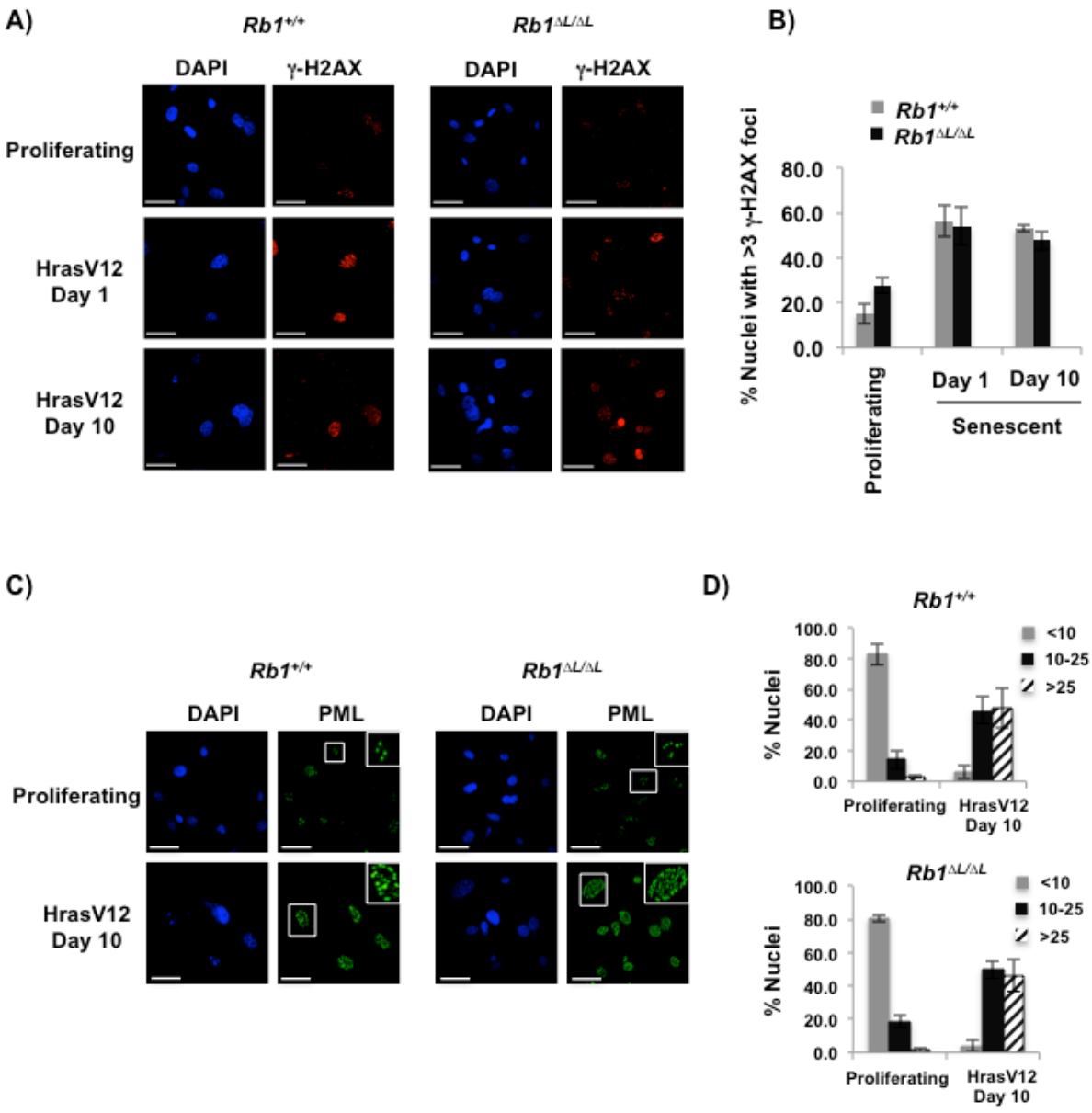

Figure 3.2 Oncogenic ras induces DNA damage and accumulation of PML nuclear bodies in both wild type and *Rb1*$^{\Delta L/\Delta L}$ cells

Asynchronously growing wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs were induced to senesce by retroviral-mediated expression of oncogenic HrasV12. After 3 days of selection, cells were re-plated and cultured for the indicated amount of time. **(A)** Immunofluorescent (IF) staining of wild type and *Rb1*$^{\Delta L/\Delta L}$ cells with γH2AX antibody (Red) to detect double strand breaks at different times after induction of senescence. Nuclei were counter stained with DAPI (Blue). **(B)** Quantification of DNA damage foci in A. The percent of nuclei with >3 γH2AX foci was compared between genotypes. **(C)** IF staining for PML nuclear bodies using a α-PML antibody (Green) and DNA counter staining with DAPI (Blue). Inset images show detailed PML staining of individual nuclei. **(D)** Quantification of the number of PML bodies per nucleus in C. The proportion of cells with fewer than 10, 10 to 25, or more than 25 PML bodies per nucleus are displayed in graphical format. Error bars represent one standard deviation from the mean, n =3. Scale bars are 50μm.

Taken together the above experiments suggest that the early events in senescence leading up to PML body assembly occur normally in *Rb1*$^{\Delta L/\Delta L}$ cells compared to wild type. This suggests that the defective enrichment of PML on E2F target gene promoters we observed is not due to decreased PML accumulation in *Rb1*$^{\Delta L/\Delta L}$ MEFs or diminished signals that induce senescence.

3.3.3 Defective senescence arrest in *Rb1*$^{\Delta L/\Delta L}$ MEFs expressing PML-IV

We next sought to determine if ectopic PML expression could rescue defective association with E2F regulated promoters in *Rb1*$^{\Delta L/\Delta L}$ fibroblasts undergoing senescence. We took advantage of the ability of PML-IV to induce senescence when overexpressed in MEFs (Bischof et al., 2002; Ferbeyre et al., 2000). We induced senescence in *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MEFs by expressing FLAG tagged PML-IV by retroviral transduction and followed the cells over a 10 day time course as above. Following selection in puromycin, cells were re-plated at low density and cultured for 10 more days to investigate the induction of senescence. Since PML induced senescence earlier than HrasV12 (Fig. 3.3A and C) we have focused on day 8 as an equivalent endpoint for these experiments. We analyzed PML-IV expressing cells for DNA synthesis, senescence associated β-galactosidase expression, and E2F target gene expression. As shown in Fig. 3.3B, FLAG-PML-IV is expressed in most cells in both the genotypes tested. 8 days post re-plating most cells had stopped proliferating as determined by BrdU and senescence associated β-galactosidase (SA-β-gal) staining (Fig. 3.3C, D and E). However, we noticed that the *Rb1*$^{\Delta L/\Delta L}$ cultures are more densely packed compared to *Rb1*$^{+/+}$ MEFs at the same time points suggesting more cell growth during this time course. To determine if *Rb1*$^{\Delta L/\Delta L}$ cells continue to proliferate following PML-IV expression before eventually exiting the cell cycle, we performed BrdU labeling at different time points after initial selection and re-plating. In response to PML-IV expression, wild type MEFs arrest as early as day 1 after re-plating and remain arrested throughout the experiment (Fig. 3.3C). In contrast, *Rb1*$^{\Delta L/\Delta L}$ cells showed elevated DNA synthesis at earlier time points as indicated by higher BrdU incorporation relative to wild type (Fig. 3.3C). However, 8 days post re-

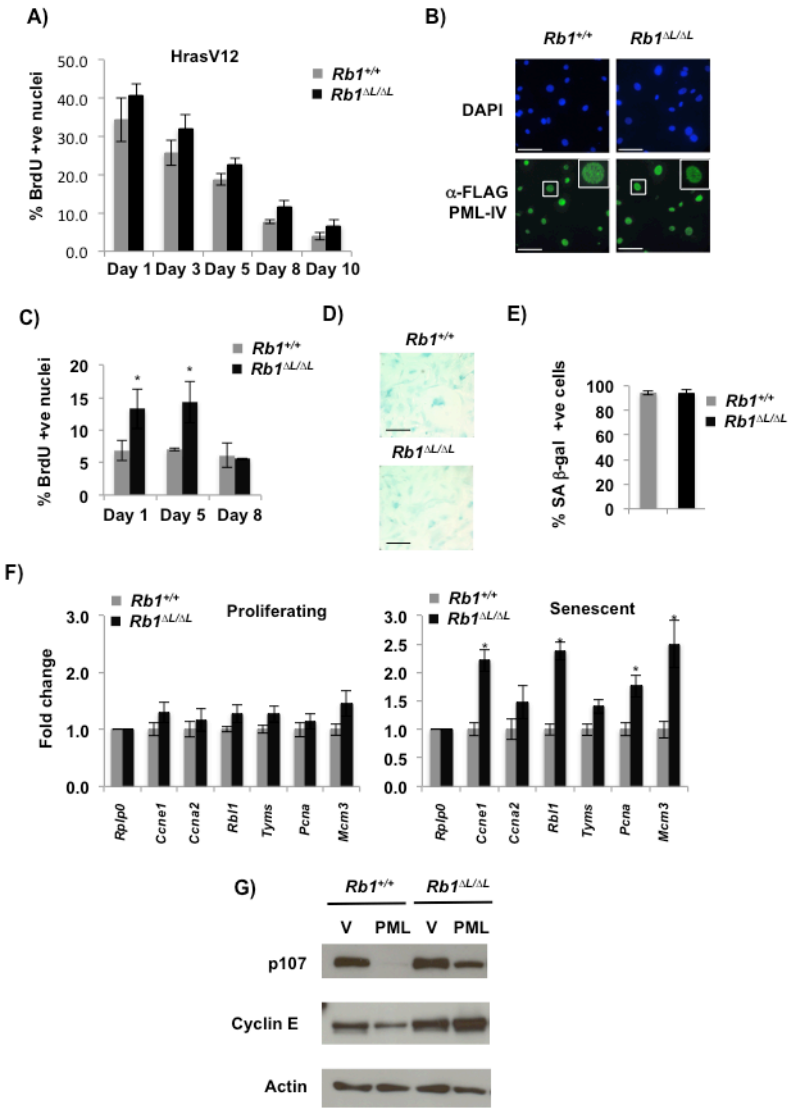

Figure 3.3 Defective senescent arrest in *Rb1*$^{\Delta L/\Delta L}$ MEFs expressing PML-IV

Asynchronously growing wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs were transduced with retroviruses expressing pBabe-HrasV12 or pBabe-FLAG-PML-IV. After 3 days of drug selection, cells were re-plated in selection medium and cultured for the indicated amount of time. **(A)** Cells of the indicated genotypes were pulsed with BrdU for 4 hours, followed by fixation and staining with α-BrdU antibodies. The % BrdU positive nuclei at the indicated time points following HrasV12 expression are plotted. **(B)** Immunofluorescent (IF) staining was performed with a α-FLAG antibody (Green) to detect FLAG-PML-IV or nuclei with DAPI (Blue). **(C)** The percentage of BrdU positive nuclei at the indicated time points following FLAG-PML-IV expression in the indicated genotypes. **(D)** PML-IV expressing cells were stained for senescence associated β galactosidase (SA-β-gal) expression 8 days after the expression of PML-IV. The number of SA-β-gal positive cells in each genotype were quantified and plotted in the right. **(E)** Quantification of E2F target gene mRNA from wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs either from proliferating (left), or PML-IV expressing cells (right). Samples are normalized to expression of the ribosomal protein gene *Rplp0*. **(F)** Western blots to determine the expression of protein products of E2F target genes (p107 and cyclin E) following empty vector (V) or FLAG-PML-IV expression (PML) are shown. Error bars indicate one standard deviation from the mean, n = 3. An asterisk indicates a statistically significant difference (P<0.05). Scale bars are 50 μm.

plating *Rb1*$^{\Delta L/\Delta L}$ cells reduce DNA synthesis to control levels (Fig. 3.3C). Furthermore, both genotypes displayed features of senescent cells at this time point as they were flat and ubiquitously positive for SA-β-gal expression (Fig. 3.3D & E). This suggests that pRB-LXCXE interactions are essential for efficient arrest of DNA synthesis and proper cell cycle exit in response to PML-IV expression, however, mutant cells still possess features of senescence in response to PML-IV.

One of the major roles of pRB in senescence is repression of E2F target genes involved in DNA replication and cell cycle advancement (Chicas et al., 2010). We next investigated if the E2F target genes are silenced in *Rb1*$^{\Delta L/\Delta L}$ MEFs in response to PML-IV expression and senescence induction. We used 8 days post re-plating as our time point for assaying E2F target gene message levels as both *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MEFs showed similar inhibition of DNA synthesis and SA-β-gal expression at this time point (Fig. 3.3C, D & E). We quantified the mRNA levels of six known E2F target genes *Ccne1* (cyclin E1), *Ccna2* (Cyclin A2), *Rbl1* (p107), *Tyms* (thymidylate synthase), *Pcna* (proliferating cell nuclear antigen) and *Mcm3* (minichromosome maintenance complex component 3) along with *Rplp0* (60S acidic ribosomal protein P0), as a control (Fig. 3.3F). In proliferating cultures, the expression levels of E2F target genes is similar in *Rb1*$^{\Delta L/\Delta L}$ MEFs compared to wild type (Fig. 3.3F left). However, in senescent cultures expressing PML-IV, 8 days post re-plating, we observed elevated expression of the E2F target genes tested in *Rb1*$^{\Delta L/\Delta L}$ MEFs relative to wild type controls (Fig. 3.3F right). Moreover, western blotting further confirmed the failure of *Rb1*$^{\Delta L/\Delta L}$ MEFs to properly repress E2F target gene expression in response to PML-IV as p107 and Cyclin E protein levels are elevated compared to controls (Fig. 3.3G).

Taken together, BrdU incorporation and E2F target gene expression analysis in response to PML-IV expression suggest that pRB-LXCXE interactions are required for proper repression of proliferative genes and efficient exit from the cell cycle. Robust induction of SA-β-gal suggests that *Rb1*$^{\Delta L/\Delta L}$ MEFs respond normally to other aspects of PML-IV induced senescence. These data suggest that PML-IV induces an incomplete state of senescence similar to HrasV12 as we have reported previously. This suggests

that PML function is critical to the switch that creates a permanent barrier to proliferation in senescence.

3.3.4 Induction of senescence signals the assembly of PML-pRB complexes that are essential for heterochromatin formation in senescence

Senescence is associated with a number of chromatin changes, and heterochromatin assembly has been suggested to play an important role (Funayama et al., 2006; Narita et al., 2006; Narita et al., 2003; Ye et al., 2007; Zhang et al., 2007; Zhang et al., 2005). Both pRB and PML have been shown to be involved in heterochromatin formation during senescence (Narita et al., 2003; Zhang et al., 2007). Consequently, we hypothesized that the defective repression of E2F target genes observed in $Rb1^{\Delta L/\Delta L}$ MEFs might be due to the inability of PML to assemble with pRB and regulate heterochromatin at these promoters.

Our analysis of PML bodies in proliferating and senescent cells in Figure 3.2C & 3.D indicates that PML bodies exist under both growth conditions. Previously, pRB was shown to bind to PML in interaction assays when over expressed in cancer cell lines (Alcalay et al., 1998). To distinguish if PML-pRB interactions are simply driven by abundance, or whether there is an active assembly process, we tested PML binding to the large pocket fragment of pRB (amino acids 379-928) fused to GST. We performed pull down experiments with GST-RB or GST-RBΔL using nuclear extracts prepared either from proliferating MEFs or those made senescent by expressing oncogenic HrasV12. As shown in Figure 3.4A GST-RB is able to pull down PML protein from senescent nuclear extracts, but not from proliferating nuclear extracts, even with relatively equal input of PML proteins. In contrast GST-RBΔL is unable to pull down PML from the same extract (Fig. 3.4A). Furthermore, GST-p107 is incapable of pulling down PML from the same extracts (Fig. 3.4B). As a control to show that the GST-RBΔL and GST-p107 proteins are functional and that equivalent amounts of extract were used in each, we stripped and re-probed the membranes with either E2F3 or E2F4 antibodies respectively. As shown in Fig. 3.4A, GST-RBΔL is able to pull down E2F3 as efficiently as wild type and

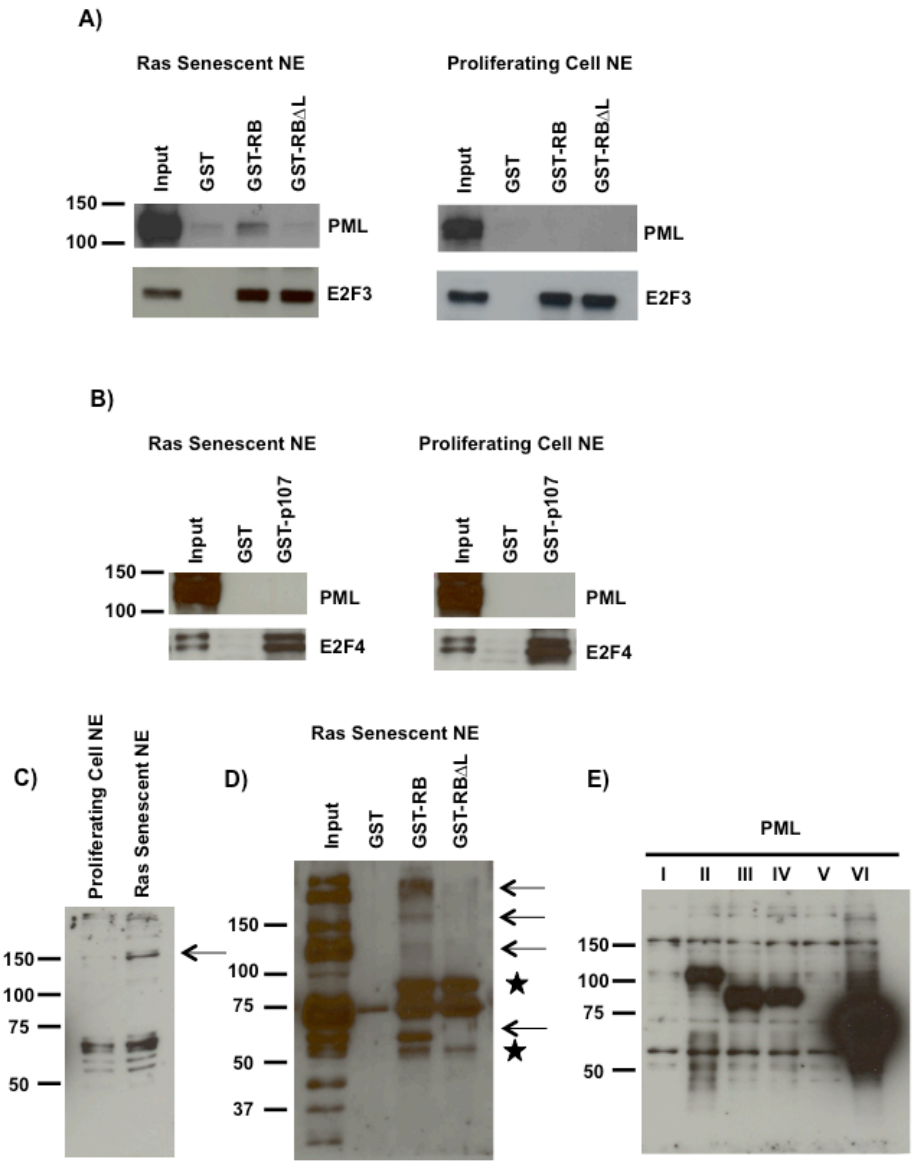

Figure 3.4 The pRB$^{\Delta L}$ mutation disrupts PML-pRB interactions during senescence

GST pull down experiments were performed using nuclear extracts from proliferating or senescent MEFs induced to senesce by expression of oncogenic HrasV12. **(A)** GST pull down using GST tagged pRB large pocket or pRB large pocket with ΔLXCXE mutations (ΔL). GST alone is used as a negative control. Pull down fractions were probed with antibodies specific to murine PML and E2F3. **(B)** GST pull down as in A using GST tagged p107 large pocket. Pull down fractions are probed with antibodies specific to either murine PML or E2F4. **(C)** Nuclear extracts from proliferating and senescent cells were analyzed by SDS-PAGE and western blotting with a pan PML antibody that recognizes numerous isoforms. The arrow indicates a differentially expressed band. **(D)** GST pull downs were carried out as in A except the blot was probed with a pan PML antibody that recognizes many PML isoforms. Arrows indicate PML species that are sensitive to ΔL mutations in pRB. Stars indicate cross reactivity with the GST-RB protein. **(E)** U2OS cells were transfected with expression constructs for each of the indicated PML isoforms. Following SDS-PAGE and western blotting, membranes were probed with the same pan PML antibody as in C to identify the migration pattern of PML isoforms.

GST-p107 is capable of pulling down E2F4 from the nuclear extracts. This indicates that GST-RB is specifically capable of interacting with PML from senescent nuclear extracts, it is dependent on LXCXE cleft interactions, and this ability is unique to pRB. These data suggest that senescence inducing stimuli such as HrasV12 signal the generation of a unique PML body that can assemble with pRB through its LXCXE binding cleft.

To expand this analysis and better understand the signal that initiates pRB-PML interactions in senescence, we used a polyclonal antibody that recognizes most isoforms of PML. First we examined PML protein expression in nuclear extracts from proliferating and HrasV12 senescent fibroblasts (Fig. 3.4C). This demonstrates the senescent dependent appearance of bands that react with PML antibodies, most notably at 150 kD molecular weight (Fig. 3.4C, marked by an arrow). In GST-RB pulldown assays we observed binding of multiple isoforms of PML with pRB in a LXCXE dependent manner (Fig.3. 4D, marked by arrows). To clarify the identity of PML proteins in this pulldown assay, we expressed FLAG tagged versions of PML I-VI individually by transfection and resolved nuclear extracts by SDS-PAGE and identified PML by western blotting (Fig. 3.4E). In agreement with previous publications, PML isoforms range from approximately 50 kD to 100 kD. Our pulldown assays reveal that some PML bands correspond to individual isoforms (eg. at 60 kD). However, it is notable that others at 150 kD and higher do not. PML is extensively modified post translationally by Sumo, among others in response to stress, which could alter their electrophoretic mobility (Jensen et al., 2001). We hypothesize that pRB-PML interactions in senescence rely on post-translational modification of known PML isoforms, to stimulate their interaction.

In order to determine the functional relevance of pRB-PML interactions in senescence we performed ChIP using α-FLAG antibodies on chromatin from cells that were induced to senesce by expressing FLAG-PML-IV. While we were able to detect FLAG-PML-IV on both *Ccne1* and *Mcm3* promoters in wild type MEFs, we could not detect a signal above background in $Rb1^{\Delta L/\Delta L}$ MEFs (Fig. 3.5A). This further suggested that PML-pRB interactions are LXCXE dependent at E2F responsive gene promoters. We next tested if PML-IV interaction with these gene promoters in $Rb1^{+/+}$ MEFs is coincident with heterochromatinization by ChIP assay. As shown in Fig. 3.5B, in $Rb1^{+/+}$

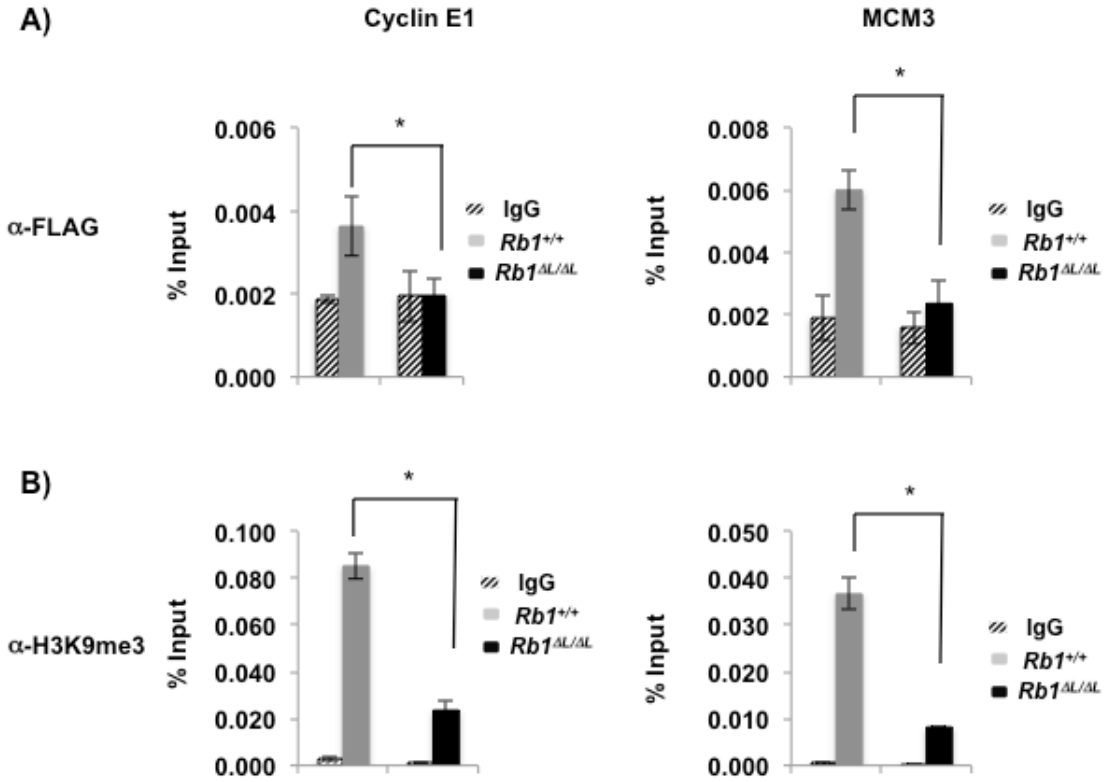

Figure 3.5 Defective enrichment of FLAG-PML-IV and heterochromatin formation at E2F target gene promoters in *Rb1*$^{\Delta L/\Delta L}$ MEFs

Asynchronously growing wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs were transduced with pBabe-FLAG-PML-IV retrovirus. After 3 days of drug selection cells were re-plated and cultured for 8 more days before processing for chromatin immunoprecipitation. **(A)** ChIP on wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs expressing Flag-PML-IV using a α-FLAG antibody or an IgG control. Real time PCR was used to amplify the immunoprecipitated DNA using primers specific to the promoter regions of *Ccne1* (left) and *Mcm3* (right). **(B)** ChIP on wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs expressing PML-IV using a α-H3K9me3 antibody or an IgG control. Real time PCR was used to amplify the immunoprecipitated DNA using primers specific to the promoter regions of *Ccne1* and *Mcm3*. Error bars indicate one standard deviation from the mean, n = 3. An asterisk indicates a statistically significant difference (*t*-test, $P<0.05$).

MEFs expressing FLAG-PML-IV, H3K9me3 is enriched at *Ccne1* and *Mcm3* gene promoters. In contrast, in *Rb1*$^{\Delta L/\Delta L}$ MEFs the enrichment of this repressive histone modification following the expression of PML-IV is drastically reduced.

The above experiments show that pRB and PML functionally interact to regulate the assembly of repressive heterochromatin at E2F target genes involved in replication and cell cycle progression. Furthermore, this interaction is mediated by the LXCXE binding cleft of pRB and pRB-PML interactions are actively stimulated by senescence.

3.3.5 Defective chromatin compaction in *Rb1*$^{\Delta L/\Delta L}$ MEFs undergoing senescence

Expression of oncogenic ras in human IMR90 fibroblasts induces widespread chromatin compaction. Individual chromosomes condense into distinct structures called senescence associated heterochromatin foci (SAHF) (Narita et al., 2003). These foci are also enriched for repressive histone modifications such as tri-methylation of histone H3 at lysine 9 (H3K9me3) a marker of heterochromatin ((Narita et al., 2003) and Figure 3.6B). However, in MEFs such global changes in heterochromatin assembly are difficult to analyze. This is because MEFs under all growth conditions show constitutive heterochromatin bodies in their nucleus that are stained by DAPI. They are comprised of the pericentromeric repeat DNA and are called chromocentres.

Interestingly, our analysis of the cells undergoing senescence suggests that the centromeric chromatin is bundled into fewer but larger chromatin bodies when compared to asynchronously growing cells (Figure 3.6A, left). Importantly, quantification of the number of the DAPI rich foci showed that senescent *Rb1*$^{\Delta L/\Delta L}$ MEFs are not as efficient in compacting their chromatin during senescence compared to the wild type cells (Figure 3.6A, right). Senescent *Rb1*$^{\Delta L/\Delta L}$ MEFs show smaller but more foci per nucleus that is very similar to what is seen in asynchronously growing cells. Moreover, the foci in the senescent *Rb1*$^{\Delta L/\Delta L}$ MEFs do not stain positive for the H3K9me3 mark, the same repressive histone modification that we found to be less enriched at the E2F target gene promoters in these cells (Figure 3.6B). This suggests that pRB plays a role in both heterochromatinization of the E2F target gene promoters and broader chromatin

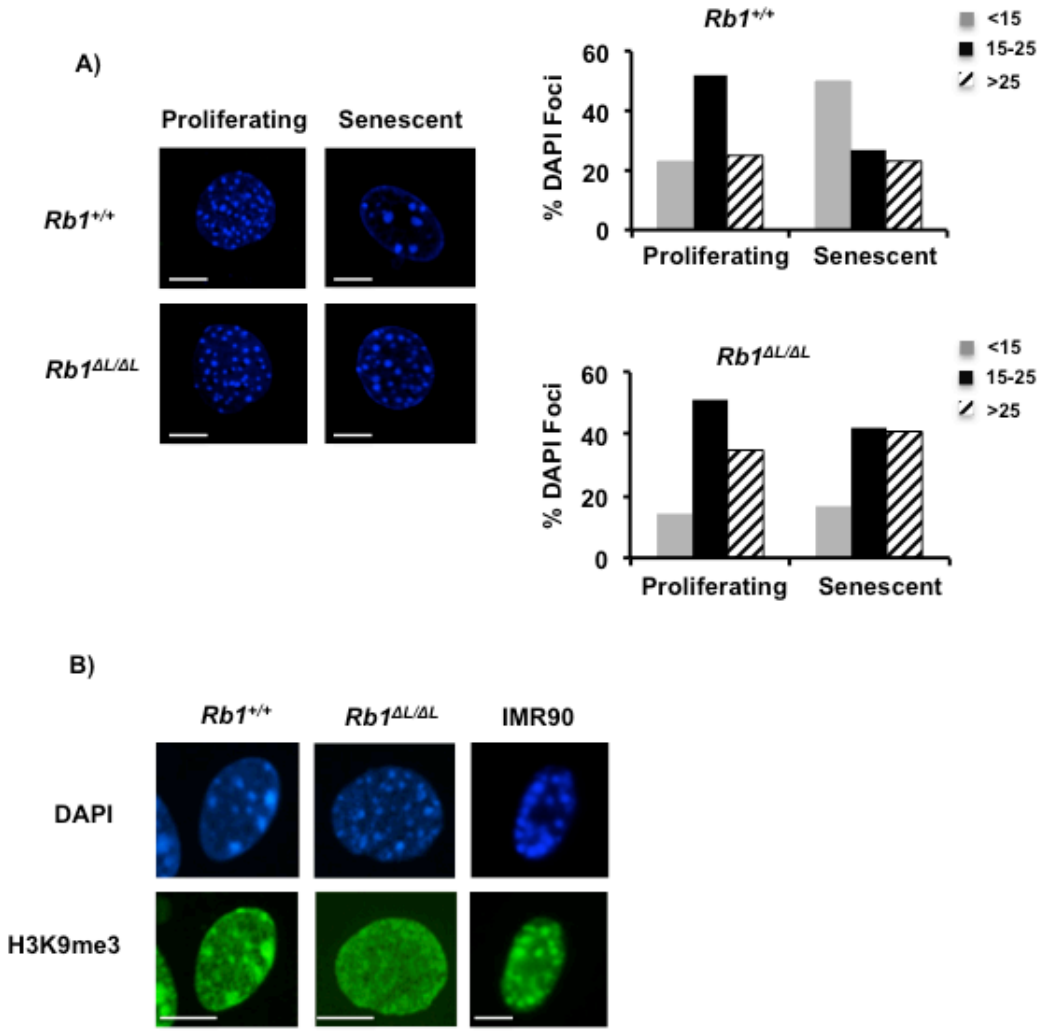

Figure 3.6 Defective chromatin compaction in *Rb1*$^{\Delta L/\Delta L}$ MEFs

Asynchronously growing wild type, *Rb1*$^{\Delta L/\Delta L}$ MEFs and IMR90 human fibroblasts were induced to senesce by retroviral mediated expression of oncogenic HrasV12. After three days of initial selection, cells were re-plated at low density and cultured for additional ten days when they become senescent. **A)** DAPI stained confocal images of asynchronously growing and senescent wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs showing chromocenters. Quantification of the number of nuclei with indicated number of chromocentres (N=2). **B)** Immunofluorescent (IF) staining of senescent wild type, *Rb1*$^{\Delta L/\Delta L}$ MEFs and IMR90cells with H3K9me3 antibody (Green). Nuclei were counter stained with DAPI (Blue). Scale bars are 10 μm.

compaction in a LXCXE dependent manner during senescence. In the future, it would be interesting to determine if these two phenomenon are related or if they are two independent functions of pRB during senescence.

3.4 Discussion

Our study demonstrates the cooperative action of PML and pRB during senescence in silencing of E2F target genes involved in DNA synthesis and cell cycle advancement. This interaction is important for heterochromatinization of these promoters as H3K9me3 deposition is severely reduced when PML and pRB are unable to assemble together at these promoters. Using a mutant version of pRB that is defective for LXCXE type interactions we demonstrated that senescence actively stimulates interactions between PML and pRB through this conserved interaction domain on pRB. The complexity of PML protein isoforms that exist in senescent cells likely contributes to their interaction with pRB. This assembly step is key to understanding the events that commit senescent cells to a permanent cell cycle arrest, and our study adds important new knowledge to ongoing work on this question.

Previous work using $Rb1^{\Delta L/\Delta L}$ MEFs and mice have allowed us to probe the circumstances where pRB uses LXCXE type interactions in cell cycle arrest. Surprisingly, pRB-LXCXE interactions are critical for stress responsive growth arrest, but not in reversible growth arrest or cell cycle arrest in development, even though each paradigm of proliferative control is pRB dependent (Andrusiak et al., 2013; Isaac et al., 2006a; Talluri et al., 2010). Notably, senescent $Rb1^{\Delta L/\Delta L}$ MEFs can re-initiate DNA synthesis in response to ectopic E2F1 expression whereas wild type cells are resistant (Talluri et al., 2010). Furthermore, serially cultured $Rb1^{\Delta L/\Delta L}$ MEFs escape senescence more readily than their wild type counterparts, suggesting that defective silencing of proliferative genes can compromise the long-term stability of senescence arrest (Talluri et al., 2010). For these reasons we have described $Rb1^{\Delta L/\Delta L}$ cells as entering into a state of partial senescence, whereby morphological features of senescence and SA-β-gal activity are typical of senescent cells, but their arrest remains reversible. A role for PML in gene silencing in growth control has been suggested, but has been less clear. First, ectopic

expression of PML inhibits growth of a number of cancer cell lines (Fagioli et al., 1998; Le et al., 1998). In addition, PML is also able to suppress the transformation of fibroblasts by activated oncogenes (Liu et al., 1995; Mu et al., 1994). Indeed, *Pml*$^{-/-}$ mice show increased susceptibility to cancer promoting agents (Salomoni and Pandolfi, 2002; Wang et al., 1998). Interestingly, in a recent study by Vernier et al. the authors showed that expression of PML in U2OS osteosarcoma cells results in the association of PML with E2F target genes and repression of their expression (Vernier et al., 2011). However, other studies have suggested that the formation of PML nuclear bodies is dispensable for induction of senescence and that the constituent proteins are key (Bischof et al., 2002). Our work reconciles these conflicting observations from two perspectives. Demonstration that endogenous PML proteins associate with E2F target promoters during the induction of senescence places PML in the right genomic location at the appropriate time to play an active role in repression of these genes by directing heterochromatin assembly. Furthermore, studies that suggest PML body formation is dispensable for senescence pre-date our description of incomplete senescence. Thereby, cells that are incapable of assembling PML bodies, but that still become SA-β-gal positive, may not have silenced E2F target genes and remain capable of cell cycle re-entry.

In our studies we demonstrate that HrasV12 expression in *Rb1*$^{\Delta L/\Delta L}$ fibroblasts triggers a similar DNA damage response as in control *Rb1*$^{+/+}$ cells. In addition, the quantity of PML bodies that are induced by HrasV12 in *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{+/+}$ cells is similar. Differences only appear when PML fails to associate with E2F target genes in senescing *Rb1*$^{\Delta L/\Delta L}$ fibroblasts. We interpret this defect to result from the failure of an active PML-pRB assembly step. We describe this event as active assembly because similar quantities of PML protein from proliferating cells fail to bind to GST-RB-LP in our assays. This interaction assay is highly relevant to PML-pRB interactions *in vivo* because it is disrupted by the same LXCXE binding cleft mutation as is present in *Rb1*$^{\Delta L/\Delta L}$ fibroblasts. Understanding how PML engages this binding site on pRB is complex. Since PML is not reported to contain an LXCXE motif it may be that the interaction is indirect and could be mediated by one or more proteins that bind to pRB through its LXCXE binding cleft. HDACs are one such potential candidate as they have

been shown to interact with both pRB and PML (Dick, 2007; Wang et al., 1998). We don't favor this interpretation because our previous studies have indicated that HDAC containing complexes interact with pRB in this type of pull down assay in a passive fashion (Isaac 2006). Based on this line of reasoning we expect that PML-pRB interactions are likely quite direct.

There are at least 6 isoforms of PML (I-VI) capable of forming nuclear bodies, and pRB has been shown to bind some isoforms preferentially (Alcalay et al., 1998). Oncogene induced senescence results in the up regulation of PML at the transcriptional and translational levels (de Stanchina et al., 2004; Ferbeyre et al., 2000). Thus, expression of PML increases in senescence, but our data indicates that relatively equal quantities of PML obtained from proliferating cells still fail to bind to GST-RB. We hypothesize that the signal to actively form pRB-PML interactions may be coincident with PML body assembly. Examination of the forms of PML present in nuclear extracts of senescent cells compared to proliferating indicates that senescence generates species of PML that are far larger than the predicted molecular weights of the largest PML isoforms. This implies that post translational modifications, such as Sumoylation, may trigger PML body assembly as well as direct the interaction with pRB.

The precise mechanism of how PML-pRB complexes inhibit the expression of E2F target genes is still unclear. Defective enrichment of repressive histone modification H3K9me3 in $Rb1^{\Delta L/\Delta L}$ MEFs provides a clue. PML might recruit and/or facilitate the incorporation of this mark to render the genes transcriptionally inert. Indeed both pRB and PML are reported to interact with Suv39h1, the enzyme capable of trimethylating histone H3K9 (Carbone et al., 2006; Nielsen et al., 2001). In addition, Suv39h1 knock out mice are defective for chromatin condensation in senescence (Braig et al., 2005). Unfortunately, we were unable to reliably detect an interaction between pRB and Suv39h1 either by co-immunoprecipitation or by ChIP in extracts from senescent MEFs. It is possible that this interaction is very transient and the conditions we used in our experiments were not conducive to detecting this interaction. Alternatively, a different enzyme may be responsible for incorporating this modification at E2F target gene promoters during senescence in a PML-pRB dependent manner. Nevertheless, ectopic

expression of PML-IV in *Rb1*$^{\Delta L/\Delta L}$ MEFs demonstrates that H3K9me3 addition to promoters might be dependent on PML-pRB interactions and the active processes described above. Previously, Nielsen et al. have demonstrated that Suv39h enzymes passively interact with GST-RB in an LXCXE cleft dependent manner. For this reason, we expect that the actual enzymatic methylation of H3K9 is downstream of a cell's commitment to enter a permanently arrested state. Our data suggests that PML-pRB interactions are likely closer to the switch that converts reversible arrest to permanent through E2F target gene heterochromatinization. Future work in this area will need to focus on the signals that assembly PML-pRB complexes as they hold the key to understanding how senescent cells become committed to permanent cell cycle arrest. Furthermore, more work is needed to better define the relationship between promoter level chromatin modifications and global chromatin reorganization during senescence.

3.5 Material and Methods

3.5.1 Cell culture

Mouse embryonic fibroblasts (MEFs) were generated from E13.5 embryos using standard procedures and cultured as previously described (Isaac et al., 2006b). The *Rb1*$^{\Delta L}$ allele encodes I746A, N750A, and M754A substitutions, and is detected by PCR genotyping as reported before (Isaac et al., 2006a). All cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with L-glutamine, streptomycin, penicillin, and 10% fetal bovine serum in a 37°C incubator with 5% CO_2. Retroviral transduction with pBABE-HrasV12 was done as reported by Serrano et al. (Serrano et al., 1997) and viruses were packaged in Bosc-23 cells. Cells infected with viruses encoding ras were pre-selected in 4μg/ml puromycin for at least 3 days before re-plating and further culturing in selection medium for 1, 5, 8 or 10 days depending on the experiment. Senescence associated ß-galactosidase (SA-β-gal) staining was performed as described (Serrano et al., 1997).

3.5.2 Immunofluorescence

Cells on coverslips were fixed in 4% PFA for 10 min at room temperature (RT), permeablized with 0.5% triton- X-100 for 5min at RT, blocked with 3% BSA/PBS for 15

min, followed by incubation with primary antibodies diluted in blocking buffer for 1hr at RT or overnight at 4^{o}C in a humidified chamber. Cells were washed in the blocking buffer 5min each for 3 times. Cells were incubated with secondary antibody diluted in blocking buffer for 1hr at RT. Cells were washed again 3 times in PBS followed by mounting on slides with mounting medium containing DAPI before analyzing by Confocal microscopy.

3.5.3 mRNA expression analysis

mRNA expression analysis of the E2F target genes was done using Quantigene Plex 2.0 reagent system from Affymetrix (Santa clara, CA) using a BioPlex200 multiplex analysis system according to Affymetrix instructions.

3.5.4 GST pull downs and Immunoprecipitations

For GST pull down assays, nuclear extracts were prepared as described before (Cecchini and Dick, 2011). GST tagged proteins were expressed in *E. coli* and purified using glutathione sepharose beads. Nuclear extracts were diluted in low-salt GSE buffer (20 mM Tris [pH 7.5], 200 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT and 0.1% NP-40) and incubated with either GST, GST-RB (large pocket, amino acids 379-928), GST- RBΔL (large pocket, with I753A, N757A, and M761A substitutions) or GST-p107 (large pocket, amino acids 385-1069) for 1 hr. Protein complexes were collected with 25 µl of Glutathione sepharose bead slurry for 1 hr. and eluted in 1X SDS PAGE sample buffer before using for western blots.

3.5.5 Chromatin Immunoprecipitations

Senescent MEFs were fixed in 1% formaldehyde in PBS for 10 min at room temperature. The reaction was stopped with Glycine at a final concentration of 0.125M. Chromatin was extracted as described before (Cortazar et al., 2011) with the following changes. Cells were sonicated for 30 min (30 s on, 30 s off, power high) using a Bioruptor sonicator (Diagenode). Diluted chromatin was pre-cleared at 4 °C for 1 h with 40 µl of a 50% slurry of magnetic Protein G Dyna beads (Invitrogen) pre bound with respective IgG. Pre-cleared chromatin was incubated with 5 µg of the antibody overnight at 4 °C

with gentle rotation. DNA was purified by using PCR purification kit from Invitrogen. Real-time PCR amplification was performed using iQSYBRGreen master mix on a BioRad CFX Connect Real Time System.

3.5.6 Antibodies

Anti-H3K9me3 (07-442), anti-γH2AX (05-636) and mouse anti-PML (MAB3738) antibodies are from Millipore. Anti-FLAG antibody (F-1804) is from Sigma. Anti-pRB (M-153), anti-PML (polyclonal) antibody H-238 (SC-5621) and anti-p107 (SC-318) antibodies are from Santa Cruz biotechnology. The anti-Cyclin-E antibody was purchased from Abcam (ab7959).

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Chapter 4

4 Mutation of the LXCXE binding cleft of pRB allows escape from oncogenic ras induced senescence and transformation *in vitro* but does not promote tumorigenesis *in vivo*

4.1 Abstract

The Retinoblastoma protein (pRB) is a key tumor suppressor that is functionally inactivated in most cancers. pRB regulates the cell division cycle and cell cycle exit through protein-protein interactions mediated by its multiple binding interfaces. The LXCXE binding cleft of pRB mediates its interactions with cellular proteins that have chromatin regulatory functions. Chromatin regulation mediated by pRB is vital for stress responsive cell cycle arrest such as oncogene induces senescence (OIS). The *in vivo* role of chromatin regulation during oncogene induced senescence and its relevance to tumor suppression is an area of active investigation. Using gene-targeted mice uniquely defective for pRB mediated chromatin regulation we investigated its role during transformation and tumor progression in response to activation of oncogenic ras. We report that the $Rb1^{\Delta L}$ mutation confers susceptibility to escape from HrasV12 induced senescence and allows transformation *in vitro* although these cells possess high levels of DNA damage. In contrast, the same mutation does not promote tumorigenesis in tumor models with activated ras mutations. Intriguingly, *KrasG12D,* $Rb1^{\Delta L/\Delta L}$ mice show delayed lung tumor formation compared to controls. This is likely due to the increased apoptosis seen in the atypical hyperplastic lesions shortly following ras activation in *KrasG12D,* $Rb1^{\Delta L/\Delta L}$ mice. Furthermore, DMBA treatment to induce ras mutations in other tissues also failed to reveal greater susceptibility to cancer in $Rb1^{\Delta L/\Delta L}$ mice. Our data suggests that chromatin regulation by pRB can function to limit proliferation, but its loss fails to contribute to cancer susceptibility in ras driven tumor models because of elevated levels of apoptosis.

4.2 Introduction

Oncogene induced senescence (OIS) has emerged as a putative tumor suppressor mechanism acting as a barrier for transformation *in vivo* (Campisi, 2001). Pre-malignant lesions such as melanocytic nevi and prostatic intraepithelial neoplasias (PIN lesions) in humans are rich in cells expressing markers of senescence, while senescent cells are rarely found in the corresponding malignant stages (Chen et al., 2005; Collado and Serrano, 2010; Michaloglou et al., 2005). In recent years several mouse models of human cancer have been generated that show activation of senescence in response to the expression of oncogenes (Collado and Serrano, 2010). Similar to human lesions, senescence in these mouse models is predominantly associated with pre-malignant stages of tumorigenesis suggesting a role for senescence in inhibiting or delaying tumor progression in response to oncogene activation *in vivo*.

Senescence is associated with activation of key tumor suppressor pathways regulated by p53 and pRB proteins (Campisi, 2005; Campisi and d'Adda di Fagagna, 2007). These two pathways coordinately inhibit the growth of pre-cancerous cells and prevent them from becoming tumors. Accordingly, disruption of these tumor suppressor pathways results in increased susceptibility to tumorigenesis in response to oncogenes (Braig et al., 2005; Chen et al., 2005; Dankort et al., 2007; Goel et al., 2009; Sarkisian et al., 2007). In mouse models, simultaneous activation of oncogenes such as HrasG12V or BRAFV600E combined with loss of p53 or p16 that act upstream of pRB resulted in escape from OIS and malignant transformation (Dankort et al., 2007; Goel et al., 2009; Sarkisian et al., 2007). Notably, enhanced tumor progression in these models strongly correlates with loss of oncogene induced senescence markers, further supporting the notion that escape from OIS is a prerequisite for malignant progression.

Senescence is a permanent cell cycle exit in which senescent cells can remain in a state of arrest throughout the life span of the organism (Campisi, 2005). The permanence of senescent arrest is partly attributed to the chromatin changes that are transcriptionally repressive and non permissible to DNA synthesis and cell division (Narita, 2007). Senescent cells form heterochromatin bodies called SAHFs (senescence associated heterochromatic foci) that are proposed to encompass and silence proliferative genes

(Narita et al., 2003). SAHFs are enriched in repressive chromatin modifications such as H3K9me3 and H3K27me3 and several chromatin associated proteins that aid in chromatin compaction and transcriptional repression (Chandra et al., 2012; Narita et al., 2003). Defects in this heterochromatin assembly pathway compromises the stability of the senescence arrest *in vitro* and are predicted to promote tumorigenesis *in vivo* (Braig et al., 2005). Intriguingly, mice lacking Suv39h1, the enzyme capable of tri-methylating histone H3K9, show defective senescence in response to oncogenic stress and increased susceptibility to tumorigenesis in the Eμ-Nras model that expresses oncogenic NrasG12D in the hematopoietic compartment (Braig et al., 2005). However, whether or not the chromatin changes during senescence have a broader tumor suppressive role *in vivo,* in response to oncogene activation in different tissues is still an open question and needs further investigation.

Lung cancer is the leading cause of cancer related deaths in humans and the most common type of lung cancer is pulmonary adenocarcinoma (Herbst et al., 2008; Jemal et al., 2008). Activated Kras mutations are the most frequently genetic alteration associated with approximately 30% of human lung adenocarcinomas (Herbst et al., 2008; Rodenhuis and Slebos, 1990). Over the last two decades a number of mouse models have been generated to model human lung adenocarcinomas in mice and they have greatly aided our understanding of the progression of the disease and the oncogene and tumor suppressor pathways involved in the process (Meuwissen and Berns, 2005). Conditional mutant models were generated harboring a latent mutant allele of *Kras* (LSL-*KrasG12D*) at its endogenous locus that can be activated sporadically in the lung cells by administering a cre expressing adenovirus (DuPage et al., 2009; Jackson et al., 2001; Meuwissen et al., 2001). This model closely mimics the human disease and has been helpful in understanding different stages of lung cancer progression.

The pRB tumor suppressor pathway is disabled in most human cancers (Sherr and McCormick, 2002). Although mutations of *RB1* are rare in lung adenocarcinomas, *CDKN2A,* the gene encoding $p16^{INK4a}$ the upstream activator of pRB pathway is frequently targeted for mutations in these tumors (Wistuba et al., 2001). $p16^{Ink4a}$ is a cyclin dependent kinase inhibitor (CKI) that inhibits D type cyclins in the G1 phase of

the cell cycle resulting in hypo-phosphorylation and activation of pRB in response to oncogenic insults. The pRB tumor suppressor protein acts by repressing E2F dependent transcription of genes involved in cell cycle progression both by direct binding and also through recruitment of chromatin regulatory complexes to these promoters (Dick, 2007). pRB is one of the major effectors of senescence and both E2F inhibition and chromatin regulatory functions are crucial for proper senescence arrest in cultured cells in response to activated oncogenes (Narita, 2007; Narita et al., 2003; Talluri et al., 2010). In the LSL-*KrasG12D* lung cancer model, lack of pRB promotes malignant transformation and enhance tumorigenesis (Ho et al., 2009). However, the contribution of different functions of pRB to its tumor suppressive role in this model is still not completely clear as complete deletion of the *Rb1* gene has many effects.

In order to investigate the role of chromatin regulation by pRB during oncogene induced senescence and tumorigenesis *in vivo*, we used a gene targeted mouse model in which the endogenous *Rb1* allele is replaced by a mutant allele $Rb1^{\Delta L}$ that is defective in binding to chromatin regulators (Isaac et al., 2006). This allowed us to study the role of chromatin regulation by pRB in isolation, as the $pRB^{\Delta L}$ is able to interact with and regulate E2F transcription factors similar to wild type. In order to investigate what affect the $Rb1^{\Delta L}$ mutation might have on tumor susceptibility *in vivo* in response to activated oncogenes, we crossed $Rb1^{\Delta L/\Delta L}$ mice with LSL-*KrasG12D* mice. We show that the $Rb1^{\Delta L}$ mutation allows escape for OIS and transformation *in vitro*. However, these cells sustain extensive DNA damage. Surprisingly, the $Rb1^{\Delta L}$ mutation delays lung tumors in the LSL-*KrasG12D* mice. $Rb1^{\Delta L/\Delta L}$; LSL-*KrasG12D* compound mutant mice show fewer lung adenomas compared to LSL-*KrasG12D* mice alone following adenovirus-cre mediated activation of oncogenic KrasG12D in the lung. Increased apoptosis in the atypical hyperplastic lesions early during tumorigenesis correlates with reduced adenomas later during tumor development. However, this defect did not affect the tumor free survival of these mice. We further show that chromatin regulation by pRB does not affect tumor free survival in a DMBA chemical carcinogen induced tumorigenesis model. Taken together our results suggest that loss of chromatin regulation by pRB facilitates escape from cell cycle arrest, but elevated levels of apoptosis prevent it from synergizing with oncogenic ras.

4.3 Results

4.3.1 The *Rb1*$^{\Delta L}$ mutation promotes escape from OIS and transformation *in vitro*

Using mouse embryonic fibroblasts (MEFs) we previously reported that the pRB LXCXE binding cleft mediated interactions are required for heterochromatin assembly and stable repression of E2F target genes during senescence (Talluri et al., 2010). In response to oncogenic HrasV12 expression, *Rb1*$^{\Delta L/\Delta L}$ MEFs undergo a defective senescence arrest that is characterized by elevated DNA synthesis and sensitivity to cell cycle re-entry in response to stimuli such as ectopic E2F1 expression. We wondered if the *Rb1*$^{\Delta L}$ mutation allows senescent *Rb1*$^{\Delta L/\Delta L}$ MEFs to escape permanently from oncogenic HrasV12 induced senescent arrest and immortalize. In order to test this, we induced senescence in asynchronously growing wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs by expressing oncogenic HrasV12 by retroviral transduction. HrasV12 expressing cells were selected for 3 days in puromycin containing medium following which they were re-plated at low density and cultured further in selection medium until they become senescent. 10 days after re-plating, most cells in both genotypes were senescent as determined by senescence associated β-galactosidase (SA-β-gal) staining and BrdU incorporation (Fig 4.1A). We then continued to culture the cells in puromycin containing medium to monitor spontaneous escape from senescence arrest. We quantified the number of spontaneous escape events by counting the distinct foci that appear in the senescent cultures. We counted a significantly higher number of foci in *Rb1*$^{\Delta L/\Delta L}$ cultures compared to the wild type cultures 3 weeks following HrasV12 expression (Fig. 4.1B). The cells in these foci have lost the characteristic features of senescence such as flattened morphology, vacuolated cytoplasm and enlarged nucleus. Interestingly, the foci in *Rb1*$^{\Delta L/\Delta L}$ cultures are bigger and often formed multilayered aggregates suggestive of loss of contact inhibition, characteristic of immortalized cell clones (Fig. 4.1C). This suggests that the *Rb1*$^{\Delta L}$ mutation confers increased susceptibility to escape from oncogene induced senescence (OIS) and this might lead to spontaneous immortalization in culture.

In order to determine if these foci are composed of immortalized cells that have

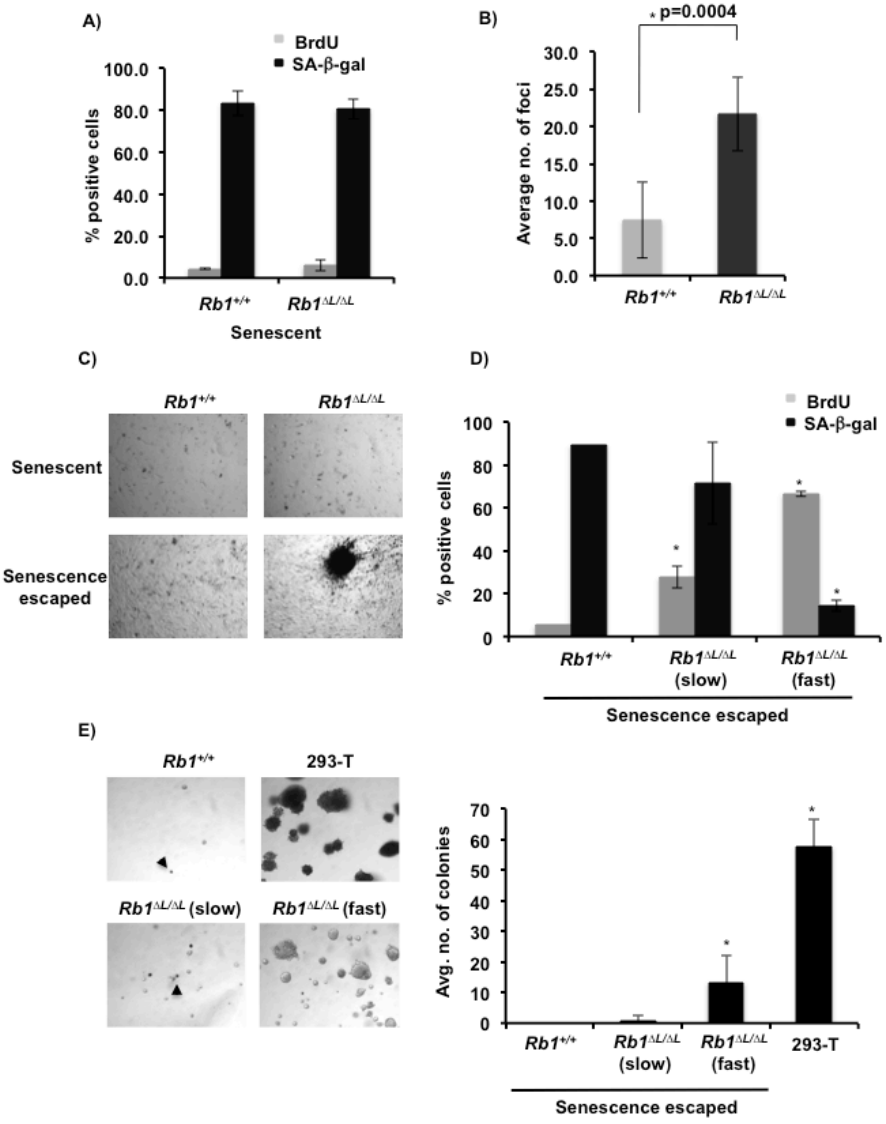

Figure 4.1 Escape from oncogenic HrasV12 induced senescence arrest and transformation in *Rb1*$^{\Delta L/\Delta L}$ MEFs

Asynchronously growing wild type and *Rb1*$^{\Delta L/\Delta L}$ MEFs are induced to senesce by retroviral mediated expression of oncogenic HrasV12. After 3 days of pre-selection cells were plated in selection medium and cultured for at least 10 days. All the cells were pulsed with BrdU for 4 hrs. A) % BrdU and SA-β-gal positive cells in senescent *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ cultures. B) Quantification of senescence escaped foci from *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ cultures. Average number of foci formed 3 weeks post pre-selection were compared between genotypes (student t-test). C) Phase contrast images of wild type and *Rb1*$^{\Delta L/\Delta L}$ cells either senescent (10 days post pre-selection) or escaped (3 weeks post pre-selection). D) % BrdU and SA-β-gal positive cells in escaped *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ clones. Escaped *Rb1*$^{\Delta L/\Delta L}$ clones were grouped into slow growing (slow) or fast growing (fast) based on their proliferative capacity as determined by BrdU incorporation and SA-β-gal staining. E) Escaped *Rb1*$^{\Delta L/\Delta L}$ clones show increased anchorage independent growth in soft agar. 293-T cells were used as a positive control. Arrowheads point toward dead cells Quantification of the number of colonies after 2 weeks of culturing in soft agar is shown on the right. Average number of colonies/field (5x) from 10 random fields. (* $p<0.05$).

permanently escaped from senescence and to study their growth characteristics we isolated cells from the foci from both $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ cultures expressing HrasV12. We sub-cultured the cells extracted from the foci in puromycin containing medium and analyzed them for proliferation and senescence using BrdU labeling and SA-β-gal staining respectively. Interestingly, most of the clones recovered from $Rb1^{+/+}$ cultures failed to survive the sub-culturing process and all of them eventually arrested with features of senescence (Fig.4.1D). In contrast, we were able to successfully sub-culture about half the clones generated from $Rb1^{\Delta L/\Delta L}$ cultures. However, these clones showed varied growth properties prompting us to categorize them into slow growing and fast growing groups (Fig. 4.1D). The slow growing clones ($Rb1^{\Delta L/\Delta L}$ slow) still showed significantly higher BrdU incorporation compared to the cells from $Rb1^{+/+}$ clone which showed growth properties very similar to senescent cultures (Fig. 4.1A and 4.1D). Strikingly, the fast growing clones ($Rb1^{\Delta L/\Delta L}$ fast) had a very high proportion of BrdU positive cells and very few cells stained positive for SA-β-gal. These cells showed highly refractive spindle shaped appearance and had an increased metabolic rate as suggested by rapid acidification of culture medium. This suggests that the $Rb1^{\Delta L/\Delta L}$ MEFs that escape from oncogenic HrasV12 induced senescent arrest have increased proliferative capability and some of them show characteristic properties of immortalized cell clones.

We next performed soft agar colony formation assays to determine if any of the escaped clones are capable of anchorage independent growth, indicative of transformation *in vitro* (Fig.4.1E). We used 293-T transformed cell line as a positive control for our assay. After two weeks of culturing, most cells from the $Rb1^{+/+}$ clone failed to grow in soft agar and many of the cells died. In contrast, cells from the fast growing $Rb1^{\Delta L/\Delta L}$ clones formed multicellular aggregates suggesting that they are capable of anchorage independent growth. Interestingly, although some of the cells from the slow growing $Rb1^{\Delta L/\Delta L}$ clones managed to form small aggregates in soft agar, we noticed cell death in these aggregates that seem to limit further growth. This suggested that in contrast to the $Rb1^{+/+}$ clones, senescence escaped $Rb1^{\Delta L/\Delta L}$ clones are capable of anchorage independent growth. However, cell death limits the *in vitro* transformation potential of these clones.

Taken together, the experiments above suggest that the $Rb1^{\Delta L}$ mutation promotes escape from oncogenic HrasV12 induced senescence and immortalization *in vitro*. Some of these clones attain anchorage independent growth potential in soft agar, suggestive of transformation.

4.3.2 The p53-p21 pathway limits the growth potential of escaped $Rb1^{\Delta L/\Delta L}$ cell clones expressing HrasG12V

We wanted to further investigate the molecular basis of differential growth properties and *in vitro* transformation abilities of the senescence escaped clones and to determine which other pathways might be limiting the transforming ability of these clones. Oncogenic ras induces hyper proliferation and replication stress resulting in increased DNA damage (Bartkova et al., 2006; Di Micco et al., 2006; Mallette and Ferbeyre, 2007). Elevated DNA damage signaling (DDR) results in the phosphorylation and activation of the p53 tumor suppressor protein. Loss of pRB function has previously been shown to result in aberrant p53 activation mediated by E2F-p19 (ARF) pathway leading to increased apoptosis limiting the transformation ability of oncogenic ras (Lara and Paramio, 2007). Interestingly, the $Rb1^{\Delta L}$ mutation leads to deregulated E2F target gene expression during senescence in response to HrasV12 (Talluri et al., 2010). So, we wondered if the p53 pathway could act as a checkpoint in the absence of pRB function in limiting the transforming ability of the escaped $Rb1^{\Delta L/\Delta L}$ clones.

We first analyzed if DNA damage signaling is intact in the $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ clones (Fig. 4.2A and B). Escaped clones from both the $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ senescent cultures showed high level of DNA damage as shown by increased γH2AX foci/nucleus (Fig. 4.2A). Strikingly, the fast growing clones have significantly higher number of foci/cell (>10) compared to both the slow growing $Rb1^{\Delta L/\Delta L}$ and the $Rb1^{+/+}$ clones. Moreover, we often found cells in the $Rb1^{\Delta L/\Delta L}$ clones where γH2AX stained the entire nucleus suggestive of drastic DNA damage. We speculate that this might be due to the high rate of proliferation in these clones causing replicative stress. We then did western blotting for γH2AX. As shown in Fig. 4.2B (right), we were able to detect γH2AX in all the clones. However, similar to IF, the fast growing $Rb1^{\Delta L/\Delta L}$ clones showed relatively higher levels of γH2AX compared to the slow growing clones suggesting elevated DNA

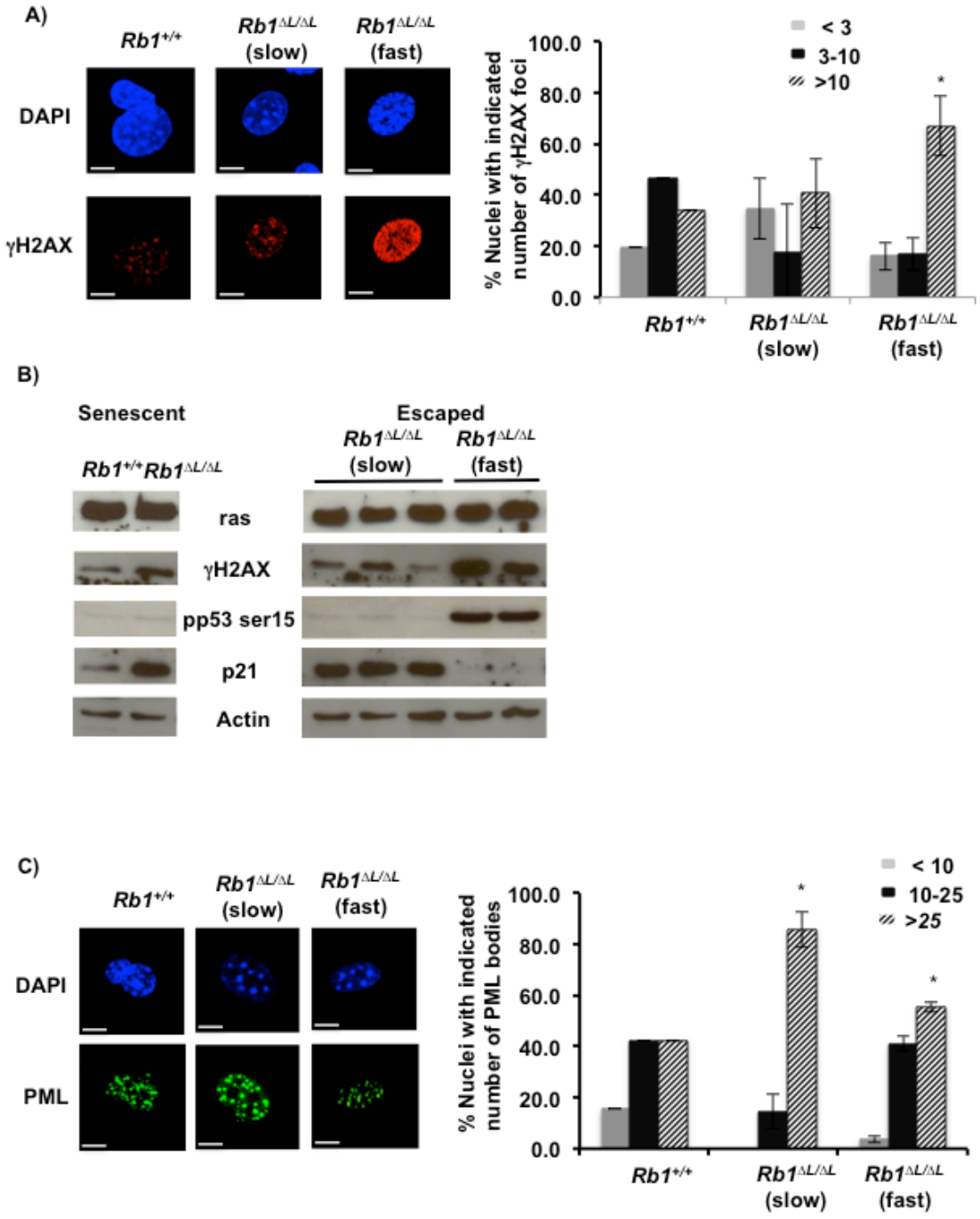

Figure 4.2 Increased DNA damage in senescence escaped *Rb1*$^{\Delta L/\Delta L}$ clones

Escaped *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ clones from Fig. 4.1 are stained with antibodies against γH2AX and PML. A) Immuno fluorescent staining of escaped *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ clones with γH2AX antibody to determine the extent of DNA damage. Quantification is shown on the right. B) Western blots for DDR proteins in senescent *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MEFs (left) and slow and fast growing *Rb1*$^{\Delta L/\Delta L}$ clones (right) C) Immuno fluorescent staining of escaped *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ clones stained with PML antibody. Quantification is shown on the right. (* Student t-test $p<0.05$). Scale bars are 10μm.

damage. All the clones however, showed similar expression of ras by western blotting (Fig. 4.2B right). We wondered if the increased DNA damage results in the activation of the p53-p21 pathway in these clones. As shown in figure 4.2B, we could detect phosphorylation of p53 at ser15 in response to the DNA damage and we interpret p53 to be active in these cells because we also detect p21 expression. Interestingly, in the fast growing $Rb1^{\Delta L/\Delta L}$ clones p53 is incapable of inducing the expression of p21 despite being phosphorylated at ser-15 suggesting that it is functionally inactive. Interestingly, senescent $Rb1^{\Delta L/\Delta L}$ MEFs also showed higher γH2AX levels and p21 induction compared to wild type cells suggesting that the $Rb1^{\Delta L}$ mutation confers susceptibility to DNA damage and this results in increased activation of the p53-p21 pathway.

Oncogenic ras expression induces the expression of PML and assembly of PML nuclear bodies in a p53 dependent manner (de Stanchina et al., 2004; Ferbeyre et al., 2000). PML has an essential role during apoptosis and cooperates with p53 to induce apoptosis in response to DNA damage (Bernardi and Pandolfi, 2003; Lara and Paramio, 2007). Consequently, we investigated the PML nuclear body formation in the escaped clones. All of the clones tested displayed an abundance of PML bodies in the nucleus that was similar to senescent cells. This suggests that the pathway upstream of PML body formation is still intact and the escape from senescence is due to defects down stream of PML body formation (Fig 4.2C).

These experiments show that the initial signaling events in response to the expression of oncogenic ras are still active in the clones that escape from senescence. The escaped clones accumulate DNA damage resulting in the activation of the p53-p21 pathway. p53 dependent apoptosis limits the growth of a subset of senescence escaped clones. *In vitro* transformation and anchorage independent growth in soft agar correlates with disruption of the p53-p21 pathway. Taken together, this indicates that defective senescence in $Rb1^{\Delta L/\Delta L}$ cells allows resumption of proliferation, but this is opposed by p53 potentially limiting transformation.

4.3.3 The *Rb1*$^{\Delta L}$ mutation does not enhance oncogenic KrasG12D driven cancer

We wondered if the ability of the *Rb1*$^{\Delta L}$ mutation to allow escape from oncogene induced senescence and immortalization in culture is sufficient to promote tumorigenesis *in vivo*. In order to test this we used a well characterized oncogenic Kras induced lung cancer model, Lox-STOP-Lox-*KrasG12D* (LSL-*KrasG12D*) (DuPage et al., 2009; Jackson et al., 2001). We crossed +/LSL-KrasG12D mice with *Rb1*$^{\Delta L/\Delta L}$ mice that are defective for LXCXE binding cleft mediated interactions to generate compound mutant mice (LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$). We then induced the expression of the latent *KrasG12D* allele in both the control mice (LSL-*Kras; Rb1*$^{+/+}$) and our compound mutant mice (LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$) using adenovirus encoding the Cre recombinase (Ad-Cre) by intranasal infection and monitored the mice following Ad-Cre infection for lung tumor free survival as well as analysis at various time points.

Deletion of pRB has been previously shown to co-operate with oncogenic KrasG12D to promote tumorigenesis in this background (Ho et al., 2009). The compound mutant mice develop more aggressive tumors and succumb to their tumors earlier than *KrasG12D* mice alone. We hypothesized that the *Rb1*$^{\Delta L}$ mutation would permit escape from senescence in lung tumor lesions, thus accelerating tumorigenesis. However, as shown in Fig. 4.3A the *Rb1*$^{\Delta L}$ mutation did not significantly alter the tumor free survival of the oncogenic KrasG12D expressing mice. The median survival was 163.5 days following activation of oncogenic KrasG12D by Ad-Cre for the LSL-*Kras; Rb1*$^{+/+}$ mice compared to183 days for the compound mutant LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice ($p>0.05$) (Fig. 4.3A). In addition, the percentage of lung weight relative to body weight at the time of euthanization was also similar between both the groups (Fig. 4.3B). This suggested that, in contrast to our *in vitro* results where *Rb1*$^{\Delta L}$ promotes escape from oncogene induced senescence and transformation, the *Rb1*$^{\Delta L}$ mutation does not promote tumorigenesis or affect the tumor free survival of mice expressing oncogenic KrasG12D *in vivo*.

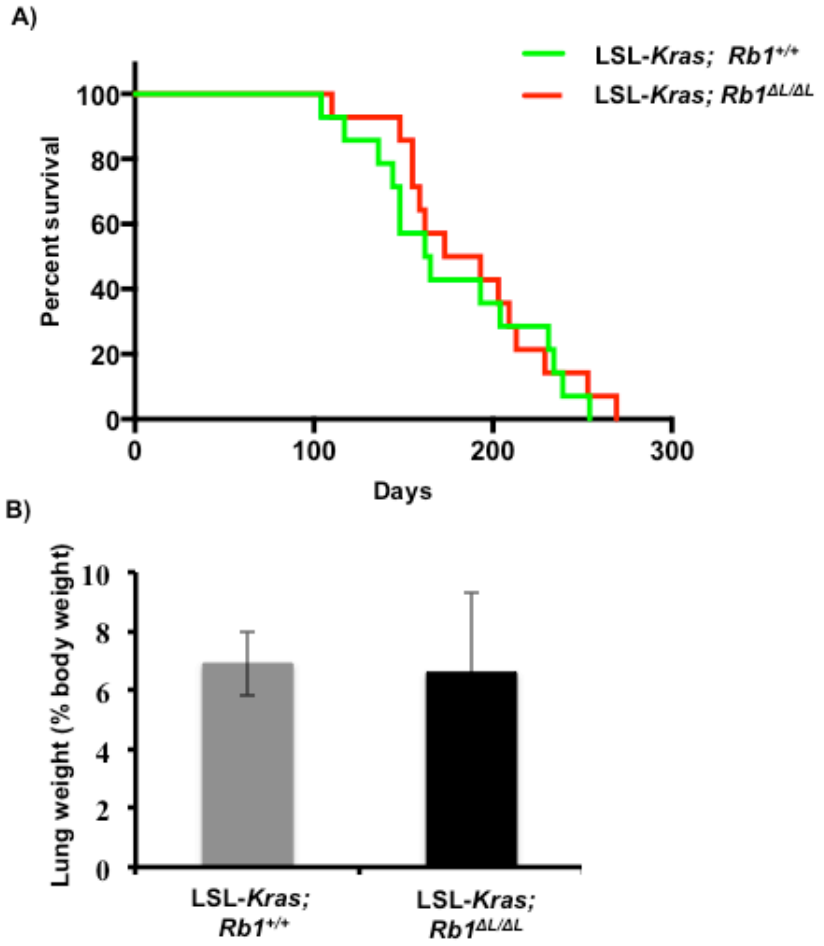

Figure 4.3 The *Rb1*$^{\Delta L}$ mutation does not affect tumor free survival in the KrasG12D lung tumor model

6-8 week old LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice were infected intra-nasally with Ad-Cre to activate the oncogenic KrasG12D. The mice were monitored over time for tumor free survival. A) Kaplan-Meier survival curves LSL-*Kras; Rb1*$^{+/+}$ (N=14) and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ (N=14) mice. The median survival age is 163.5 and 183 days respectively (p=0.636, log rank test). B) Mean lung weight represented as % total body weight at the time of death.

4.3.4 Fewer lung tumor lesions in *Rb1*$^{\Delta L/\Delta L}$ mice expressing oncogenic KrasG12D

Oncogene induced senescence is widely believed to act as a barrier to transformation and cancerous growth *in vivo* and expression of oncogenic ras has been shown to activate senescence thereby limiting tumorigenesis in mouse models (Collado and Serrano, 2010). Our *in vitro* results suggested that cells from *Rb1*$^{\Delta L/\Delta L}$ mice have defective senescence allowing them to escape from this arrest and transform. However, this did not lead to enhanced tumor susceptibility *in vivo*. As a result, we wanted to further investigate tumorigenesis in the LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice more closely by quantifying the number of lesions that develop in response to KrasG12D activation.

We measured the number of lung tumor lesions from both the LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice post activation of oncogenic KrasG12D by Ad-Cre recombinase. At 12 weeks post Ad-cre infection, we were able to detect different types of lesions as reported in the literature such as atypical adenomatous hyperplasia (AAH), adenoma, adenocarcinoma and epithelial hyperplasia of the bronchioles (EHB) in both the experimental groups (Fig 4.4A) (Jackson et al., 2001). AAH and adenoma are benign lesions and are considered precursors for adenocarcinoma, which is a malignant state. Strikingly, as shown in Fig. 4.4B, lungs from LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice showed significantly fewer adenomas compared to LSL-*Kras; Rb1*$^{+/+}$ controls. We wondered if the reduced number of adenomas we see in LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice is due to their hastened progression to adenocarcinoma. However, we did not notice any concomitant increase in the number of adenocarcinoma in LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice compared to controls. We also did not notice any difference in the number of AAH, the precursor lesions of adenoma between the two groups at this time point. Interestingly, at the time of harvesting the lungs for histology we also observed that the lungs from LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice are often smaller in size compared to the control LSL-*Kras; Rb1*$^{+/+}$ mice (Fig. 4.4 A). This suggested to us that, contrary to our original prediction, the *Rb1*$^{\Delta L}$ mutation might be negatively affecting the growth of KrasG12D induced tumors. We then performed the same analysis at an earlier time point at 6 weeks post Ad-Cre infection, to rule out the possibility that the decreased adenoma incidence in

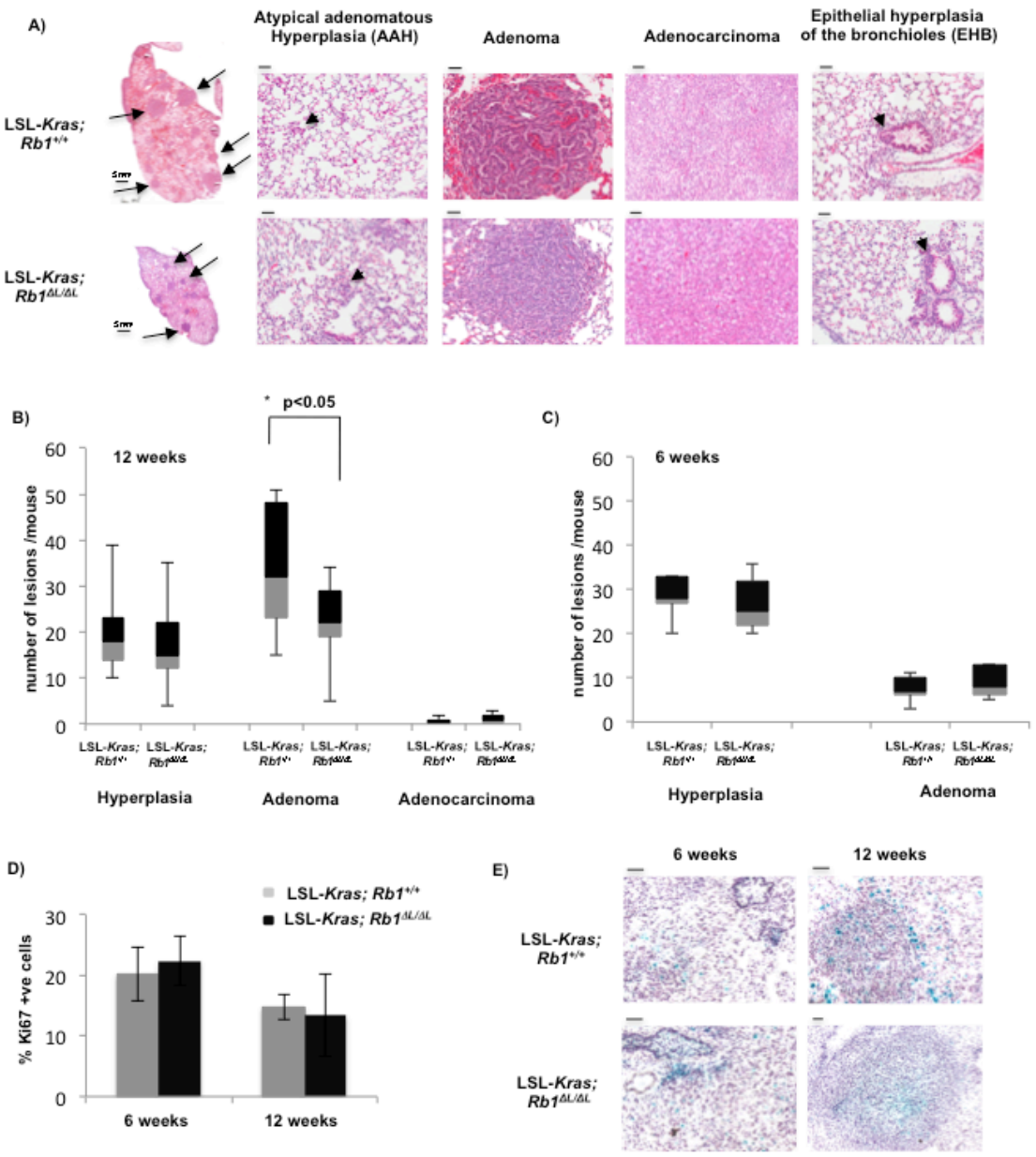

Figure 4.4 Fewer lung tumor lesions in *Rb1*$^{\Delta L/\Delta L}$ mice expressing KrasG12D

6-8 week old LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice were infected intra-nasally with Ad-Cre to activate the oncogenic KrasG12D. The mice were analyzed for lung tumors at 6 and 12 weeks post Ad-Cre infection. A) Haematoxylin and Eosin stained lung sections from 12 week old mice of mentioned genotypes showing different lung tumor lesions. Arrow heads points towards the smaller lesions. Scale bars are 50μm unless otherwise specified. B) Quantification of the different lung tumor lesions 12 weeks post Ad-Cre infection. (* Student t-test). Box and whisker plots show 25th percentile, median and 75th percentiles. Whiskers show highest and lowest values in the group C) Same as B at 6 weeks post Ad-Cre infection. D) Quantification of Ki67 positive cells in the adenomas from LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ lungs at indicated time points. E) Senescence associated β-galactosidase staining of cryo-sections from LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ lungs at indicated time points. Sections were counter stained with Haematoxylin. Scale bars are 50 μm unless stated otherwise.

LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice was due to fewer early lesions in response to oncogenic KrasG12D activation following Ad-Cre infection. The majority of the lesions seen at the 6-week time point are atypical adenomatous hyperplasia (AAH) and we noticed only a few adenomas at this stage (Fig. 4.4C). Quantification of the number of lesions showed a similar number of hyperplastic lesions between control and the LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ groups at this time point. This suggests that the activated oncogenic KrasG12D is able to initiate early lung lesions similarly between both the genotypes.

In order to investigate the possible cause for the decreased number of adenomas in LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice we first tested the proliferation rate of the tumor cells between the two genotypes at both 6 weeks and 12 weeks following the activation of KrasG12D. We used Ki67 as a marker for proliferation and SA-β-gal staining as a marker for senescence in the tumors. At both time points tested the number of Ki67 positive cells in the adenomas is very similar between the two experimental groups suggesting that the tumor cells are proliferating at a similar rate in both groups (Fig. 4.4D). Furthermore, we also detected similar senescence staining in the adenomas from LSL-*Kras; Rb1*$^{+/+}$ and the LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice. This suggested that there is no defect in the proliferation of tumor cells in the LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice and activation of oncogenic KrasG12D induces senescence in these lesions similar to those in LSL-*Kras; Rb1*$^{+/+}$ controls.

4.3.5 Increased apoptosis in atypical adenomatous hyperplasia (AAH) lesions from *Rb1*$^{\Delta L/\Delta L}$ lungs expressing KrasG12D

Oncogenic ras expression induces apoptosis through p53 or pRB-E2F1 pathways (Fikaris et al., 2006). Our *in vitro* results suggested that the clones that escaped senescence have high levels of DNA damage as shown by increased γH2AX staining and activation of the p53-p21 pathway. We wondered if escape from senescence because of the *Rb1*$^{\Delta L}$ mutation results in increased cell death by apoptosis *in vivo*, which could potentially explain the reduced number of adenomas that we observe in LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice.

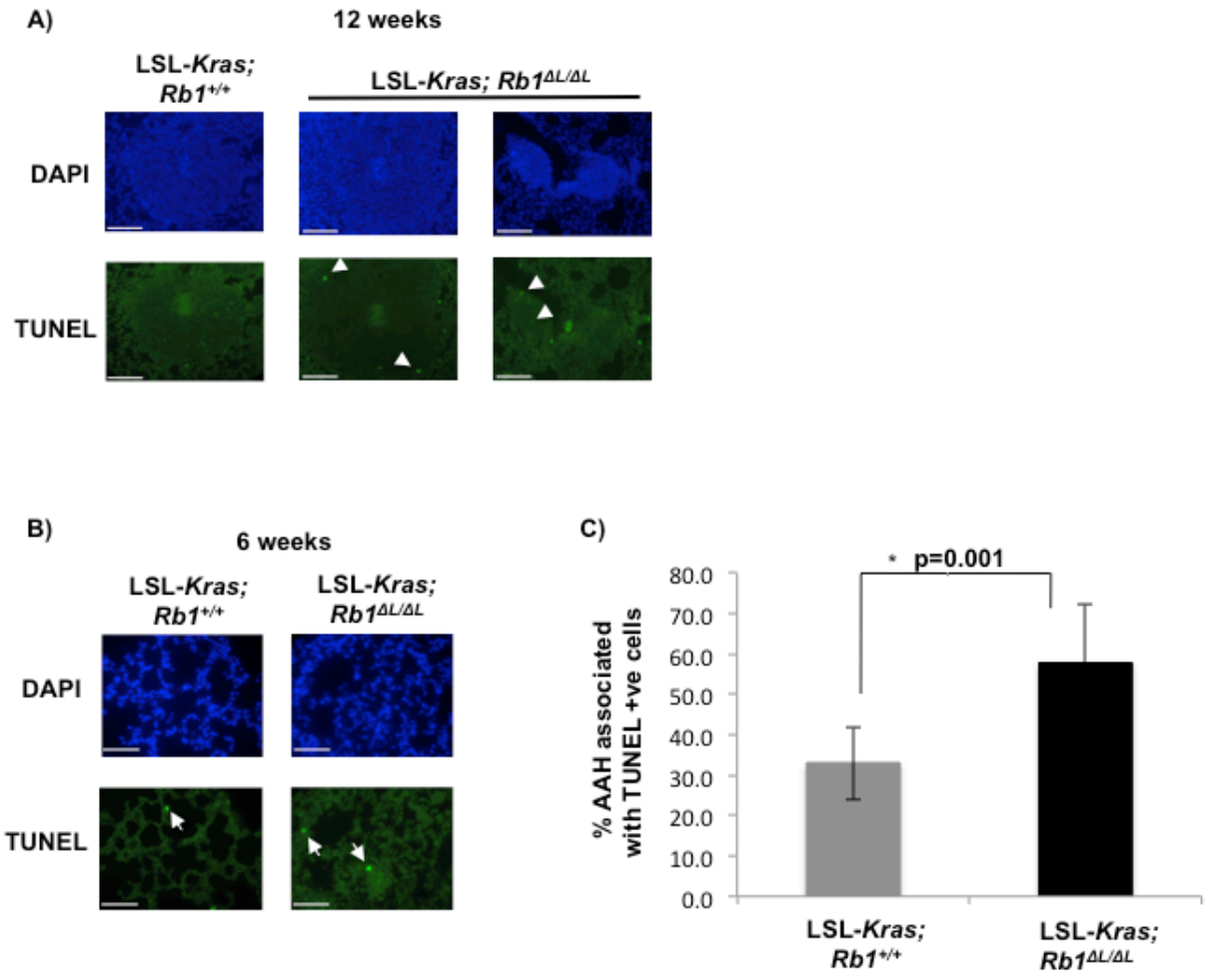

Figure 4.5 Elevated apoptosis in atypical adenomatous hyperplasia (AAH) lesions from lungs of *Rb1*$^{\Delta L/\Delta L}$ mice expressing KrasG12D

6-8 week old LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice were infected intra-nasally with Ad-Cre to activate the oncogenic KrasG12D. Paraffin embedded lung sections at 6 or 12 weeks post Ad-Cre infection were processed for TUNEL staining using *in situ* cell death detection kit from Roche. Arrowheads point to TUNEL positive cells. A) Representative TUNEL stained lung sections from LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice 12 weeks following Ad-Cre infection. DAPI is used to stain the nuclei. B) Representative TUNEL stained lung sections 6 weeks following Ad-Cre infection. DAPI is used to stain the nuclei. C) Quantification of % TUNEL positive cells in B that are associated with atypical adenomatous hyperplasia (AAH) lesions in the LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ lungs (* t-test). Scale bars are 50 μm.

We searched for evidence of apoptosis in the adenoma lesions in both *Rb1* genotypes. We performed TUNEL staining on lung sections from LSL-*Kras; Rb1*$^{+/+}$ and LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice at different time points following KrasG12D activation. We noticed very few TUNEL positive cells in the adenomas from the LSL-*Kras; Rb1*$^{+/+}$ mice (Fig. 4.5A) at 12 weeks post activation of KrasG12D, suggesting absence of cell death in these lesions. However, some of the adenomas from LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice showed TUNEL positive cells at this time point suggesting cell death. We hypothesized that apoptosis might be activated early during tumor development in response to activated ras and this limits the progression of these lesions. Therefore, we searched for apoptosis in lung sections of mice 6 weeks post activation of KrasG12D by TUNEL staining. We counted the TUNEL positive cells from at least 10 random fields from each lung section and quantified how many of these are associated with early hyperplastic lesions i.e. AAH (Fig. 4.5B). We saw a significantly higher number of TUNEL positive cells that are associated with AAH lesions in the LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice compared to the controls. This suggested that cell death by apoptosis may play a role in inhibiting tumor progression in this model and that the pRB$^{\Delta L}$ mutation is exacerbating this cell death. This would result in fewer AAH lesions progressing to the adenoma stage. This could in part explain why we see lower numbers of adenomas in the LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice compared to controls at 12 weeks post activation of KrasG12D.

4.3.6 *Rb1*$^{\Delta L}$ mutation does not exacerbate tumorigenesis in a DMBA induced chemical carcinogenesis model

To complement the *KrasG12D* mice study and to investigate whether tissue specificity plays a role on the affect of *Rb1*$^{\Delta L}$ mutation on the tumor susceptibility, we used 7,12-dimethyl benz[a]anthracine (DMBA) induced chemical carcinogenesis to induce tumors. Administration of DMBA has been shown to cause ras mutations and promote tumorigenesis in several tissues in mouse models (Pazzaglia et al., 2001; Quintanilla et al., 1986). We wanted to investigate if the *Rb1*$^{\Delta L}$ mutation promotes tumorigenesis in response to DMBA treatment. We treated 6 week old *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mice with DMBA once a week for four weeks by oral gavage and monitored the mice for tumors (Fig. 4.6A).

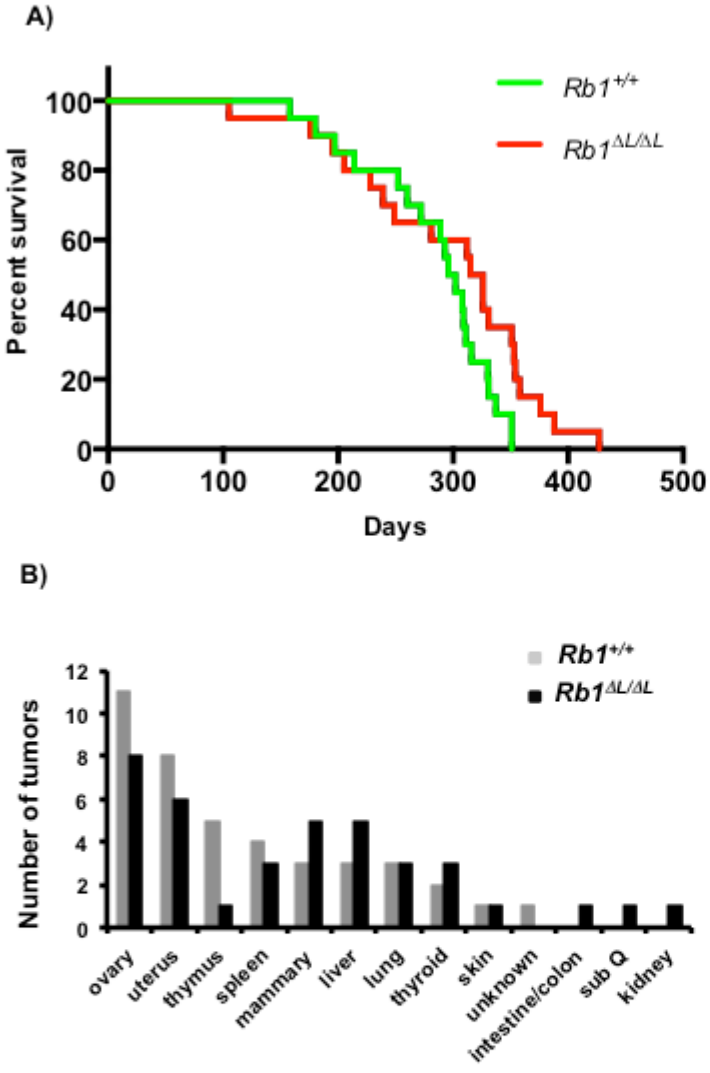

Figure 4.6 The *Rb1*$^{\Delta L}$ mutation does not affect tumor free survival in DMBA induced carcinogenesis model

6-8 week old *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mice were dosed with 1mg/ml of DMBA in canola oil as vehicle, weekly for 4 weeks A) Kaplan-Meier survival curves of *Rb1*$^{+/+}$ (N=20) and *Rb1*$^{\Delta L/\Delta L}$ (N=20) mice treated with DMBA. The median survival age is 299 and 320.5 days respectively (p=0.0537, log rank test). B) Quantification of the tumors in various tissues at the time of death in *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mice treated with DMBA.

As seen in Fig. 4.6A, the $Rb1^{\Delta L}$ mutation did not significantly alter the tumor free survival of the mice in DMBA carcinogen induced tumor model. The over all tumor free survival rate was similar between the genotypes with 95% of $Rb1^{+/+}$ mice and 100% $Rb1^{\Delta L/\Delta L}$ mice succumbing to tumors. The median tumor free survival is 320.5 days for the $Rb1^{\Delta L/\Delta L}$ mice compared to 299 days for the $Rb1^{+/+}$ mice (p=0.0537). Necropsy of the mice from both the groups showed tumor incidence in a number of tissues (Fig. 4.6B). In addition, we did not observe significant difference between the two groups in terms of disease site as both genotypes developed a broad range of cancer types.

Given the preponderance of ras mutations that are known to be caused by DMBA, when considered along with the KrasG12D lung cancer model, it suggests that the $Rb1^{\Delta L}$ mutation does not promote tumorigenesis in a mutant ras background irrespective of the tissue type or the mode of activation of the ras oncogene.

4.4 Discussion

Our study shows that mutation of the LXCXE binding cleft that disrupts chromatin regulation by pRB is sufficient for escape from oncogene induced senescence and transformation *in vitro.* Interestingly, the $Rb1^{\Delta L}$ mutation does not promote tumorigenesis *in vivo* but instead, reduces tumorigenesis in the *KrasG12D* lung cancer model by negatively affecting early tumor progression.

We think there are a number of explanations for the discrepancy we observe between our *in vitro* findings and *in vivo* results. One potential explanation could lie in the obvious differences in cell types between our experiments. Unlike the lung alveolar pneumocytes, mouse embryonic fibroblasts (MEFs) are not terminally differentiated cells. It is thus possible that MEFs in culture are more readily immortalized whereas additional checkpoints that prevent transformation of the differentiated cells exist *in vivo.* The increased apoptosis we observe in the primary AAH lesions that are precursors of adenomas and adenocarcinomas suggests this might be the case. It is also interesting to note that MEFs do not undergo apoptosis as robustly and rather activate senescence in response to stress. Defective heterochromatinization and deregulation of cell cycle genes during senescence, as a result of the $Rb1^{\Delta L}$ mutation, seems to be sufficient for random

cells to escape from this arrest and immortalize *in vitro*. However, the relative contribution of apoptosis and senescence during tumor suppression *in vivo* is not fully understood. Even though both phenomenon are known to be tumor suppressive and are regulated by the same tumor suppressor networks, how and at what stage during tumor progression they coordinate to suppress tumorigenesis is still not completely understood. We show that senescence is activated in the lungs in response to the activation of oncogenic KrasG12D at a very early stage, and benign lesions stain positive for SA-β-gal suggesting that senescence does play a role in suppressing tumor progression in this model. From this perspective it appears that oncogenic KrasG12D expression activates both apoptosis and the senescence pathway. Based on our data, cell death by apoptosis prevents early lesions from progressing to the later stages. Senescence further acts by suppressing the growth of the benign lesions thereby preventing or delaying tumor progression.

The cause of the increased apoptosis we observe in the LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ mice is not clear. A recent report has suggested a complex interplay between heterochromatin assembly during senescence and suppression of DNA damage response (DDR) signaling (Di Micco et al., 2011). The authors show that disruption of heterochromatin in oncogene expressing cells increases DDR signaling leading to apoptosis. So, it is possible that defective heterochromatinization during senescence as a result of the *Rb1*$^{\Delta L}$ mutation might be exacerbating DDR signaling and induce apoptosis in these lesions early during tumorigenesis in our model. However this could be context specific as defective heterochromatinization in mice lacking Suv39h1, the enzyme capable of tri-methylating histone H3K9, show increased susceptibility to lymphoma development in the Eμ-N-ras model (Braig et al., 2005). In short, loss of heterochromatin assembly can't universally antagonize cancer progression as we've shown here.

Our studies using the *KrasG12D* model and the DMBA chemical carcinogenesis model show that *Rb1*$^{\Delta L}$ mutation does not promote tumorigenesis or affect overall survival of the mice. Previous studies done using the *Rb1*$^{\Delta L/\Delta L}$ mice also suggested a context specific role for the LXCXE binding cleft during tumorigenesis (Coschi et al., 2010; Francis et al., 2011). The *Rb1*$^{\Delta L}$ mutation co-operates with p53 loss to hasten

tumor formation in mice (Coschi et al., 2010). The tumors in the compound mutant mice are more genomically unstable and are also more aggressive. Also, in a mammary tumorigenesis model the *Rb1*$^{\Delta L}$ mutation exacerbates the tumor phenotype in the Wap-p53 (R172H) transgenic background. However, in the same study they found that the *Rb1*$^{\Delta L}$ mutation does not affect Neu oncogene induced mammary tumors. This suggests that tumor suppression by the LXCXE binding cleft of pRB is highly context specific. While the *Rb1*$^{\Delta L}$ mutation enhances tumorigenesis when combined with p53 loss the same mutation does not cooperate with oncogene activation in the receptor tyrosine kinase (RTK)-ras pathway to promote tumor formation. This might suggest that even though the *Rb1*$^{\Delta L}$ mutation results in deregulated cell cycle gene expression and defective cycle arrest, *in vivo*, the p53 pathway might act as an additional barrier to suppress tumorigenesis. Increased apoptosis seen in our LSL-*Kras; Rb1*$^{\Delta L/\Delta L}$ tumors also seems to supports this hypothesis. Tumor progression in these models might require additional disruption of the p53 tumor suppressor pathway. Future experiments using gene targeted mouse models with subtle mutations like ours will help our understanding of the complex relationship between different tumor suppressor and oncogene networks that exist *in vivo*.

4.4.1 Material and Methods

4.4.2 Mice

The generation of *Rb1*$^{\Delta L/\Delta L}$ mutant mice has been described before (Isaac et al., 2006). LSL-*KrasG12D* mice (Jackson et al., 2001) were obtained from NCI mouse repository in a B6.129 background and maintained as heterozygotes and were bred to the *Rb1*$^{\Delta L}$ mice also in B6.129 background. Genotyping methods and PCR primers were provided by the suppliers, or are as outlined by Isaac, et al. All animals were housed and handled as approved by the UWO animal use subcommittee (protocol 2007-058) and Canadian Council on Animal Care (CCAC) guidelines.

4.4.3 Ad-Cre infection

Ad-Cre was administered by intranasal instillation as described before (DuPage et al., 2009; Jackson et al., 2001). We infected mice with $5x10^6$ infectious particles of Ad-Cre in 75 μl volume per mouse.

4.4.4 Histology

Lungs were fixed in formalin for 48hrs before embedding in paraffin for staining with Haematoxylin and Eosin. For immunohistochemistry, formalin fixed, paraffin embedded tissues were deparrafinized in xylenes followed by rehydration by serial washes in 100%, 95%, 70% ethanol and water. Antigen retrieval was done by boiling the sections in a pressure cooker for 15 min in 10 mM sodium citrate buffer pH6.0. TUNEL staining was performed using *in situ* cell death detection kit from Roche as per the manufacturers instructions (Cat. No. 11 684 795 910). Tissues for SA β-gal staining were fixed in optimum cutting temperature (OCT) compound and embedded for cryo-sectioning.

4.4.5 Senescence β-galactosidase staining on tissues

Tissue sections were processed immediately after cryo-sectioning by fixing them in 0.5% glutaraldehyde/PBS for 15 minutes followed by O/N incubation in SA-β-gal staining buffer (40 mM citric acid/sodium phosphate buffer at pH 6.0 containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2 and 1mg/ml X-gal) at 37^{o}C in a humidified chamber. Sections were washed with PBS before sealing with cover slips using Vectamount mounting medium.

4.4.6 Microscopy

Haematoxylin and Eosin and antibody stained sections are scanned using Aperio Scan Scope CS2 system. Scanned sections were analyzed using Aperio Image Scope viewer software. Lesions were manually counted and graded based on the recommendations of the Nikitin et al. and mouse models of human cancer consortium (Nikitin et al., 2004).

4.4.7 Cell culture

Mouse embryonic fibroblasts (MEFs) were generated from d13.5 embryos using standard procedures and cultured in DMEM with 10% FBS and antibiotics(Hurford et al., 1997). Retroviral transduction with pBABE-HrasV12 was as reported by Serrano et al. (Serrano et al., 1997) and viruses were packaged in Bosc-23 cells. Cells infected with viruses encoding HrasV12 were pre-selected in 4μg/ml puromycin for at least 3 days before processing for further experiments. Senescent cells prepared by this method were

allowed to senescence for at least 10 days following retroviral infection. Senescence associated β-galactosidase (SA-β-Gal) staining was performed as described before (Serrano et al., 1997). Escaped clones were continuously sub-cultured in standard medium with 4μg/ml puromycin and passaged every 3 days.

4.4.8 Soft agar colony formation assay

6 well dishes were coated with a bottom layer of 1.5 ml 0.7% low melting agarose in DMEM with 10% FBS and antibiotics. $3x10^4$ cells were resuspended in 1.5 ml of 0.35% low melting agarose in DMEM with 10%FBS and antibiotics and added as the top layer to the 6 well plates. All genotypes were tested in triplicate. Cells were allowed to grow in soft agar for 2 weeks before counting the number of colonies formed.

4.4.9 Antibodies

Anti-γH2AX (05-636) and anti-PML (MAB3738) antibodies are from Millipore. Anti actin (A2066) antibody is from Sigma. Anti-phospho-p53-ser15 (9284) antibody is from cell signaling. Anti pan-ras antibody (FL-189) is from Santa cruz. Anti-p21 (AB-4)(OP-76) antibody was purchased from Calbiochem.

4.4.10 Immunofluorescence

Cells were fixed in 3% PFA for 10 min at room temperature (RT) and permeablized with 0.5% triton- X-100 for 5min at RT. Cells were blocked with 3% BSA/PBS for 15 min at RT followed by incubation with primary antibodies diluted in blocking buffer (1:300 PML, 1:200 γH2AX) overnight at 4^0C in a humidified chamber. Cells were washed in the blocking buffer 3 times for 5min each. Cells were incubated with Alexa-fluor conjugated secondary antibodies diluted in blocking buffer (1:4000) for 1hr at RT. Cells were washed again 3 times in PBS followed by mounting on slides with mounting medium containing DAPI before analyzing by Confocal microscopy.

4.5 References

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Chapter 5

5 Discussion

5.1 Summary and significance of current work

The primary goal of this study was to investigate how the recruitment of chromatin regulating proteins by pRB influences mammalian development and disease. In order to investigate this, I used a gene targeted mouse strain in which mutations in the *Rb1* gene disrupt only LXCXE binding cleft mediated interactions.

In chapter 2 I investigated whether pRB-LXCXE interactions are required under all G1 arrest circumstances to regulate chromatin and block cell cycle as has traditionally been suggested in the field, or if they have a more specialized role during stress response. I studied the role of pRB-LXCXE interactions in two permanent cell cycle exit paradigms terminal differentiation and cellular senescence. I investigated cell cycle exit in skeletal muscle and retinal neurons, two long lived cell types, and compared it with cell cycle exit during senescence using cells derived from $Rb1^{\Delta L/\Delta L}$ mice. I showed that there is defective inhibition of DNA synthesis specifically during senescence in $Rb1^{\Delta L/\Delta L}$ cells, but not during terminal differentiation. Senescent $Rb1^{\Delta L/\Delta L}$ fibroblasts fail to heterochromatinize E2F responsive genes as shown by a deficiency in enrichment of H3K9me3. This causes de-repression of cell cycle genes and leaves the $Rb1^{\Delta L/\Delta L}$ cells susceptible to proliferative stimuli. However, analysis of the same genes in terminally differentiated muscle tissue from $Rb1^{\Delta L/\Delta L}$ mice showed normal abundance of heterochromatin marks and proper silencing of these genes similar to wild type. Unexpectedly, this study revealed that pRB uses LXCXE interactions specifically to regulate a stress responsive growth control mechanism that is distinct from cell cycle exit in terminal differentiation during development.

In chapter 3 I further explored the mechanism of pRB mediated stable silencing of the E2F responsive cell cycle genes during senescence. My experiments show that during oncogene induced senescence endogenous PML is recruited to E2F responsive cell cycle

gene promoters in a pRB-LXCXE dependent manner. Interestingly, during PML induced senescence the E2F target genes fail to be repressed in *Rb1*$^{\Delta L/\Delta L}$ MEFs. Overexpressed PML is also unable to bind to the cell cycle gene promoters in *Rb1*$^{\Delta L/\Delta L}$ cells. Importantly, this is associated with a striking reduction in the enrichment of the repressive histone mark H3K9me3 at these promoters. Taken together, the experiments in chapter 3 suggest a model in which pRB interacts with PML in a LXCXE cleft dependent manner specifically during senescence. This pRB-PML complex is important for heterochromatinization and stable silencing of E2F target cell cycle genes during senescence.

In chapter 4 I investigated if chromatin regulation by pRB is required for preventing oncogenic ras induced transformation *in vitro* and tumorigenesis *in vivo*. I showed that the LXCXE interactions of pRB are necessary to prevent escape from OIS and transformation *in vitro.* However, *in vivo, Rb1*$^{\Delta L/\Delta L}$ mice expressing oncogenic KrasG12D develop fewer lung tumors compared to the control mice. My results suggest that increased apoptosis in hyperplastic lesions early during tumorigenesis might be one of the reasons for reduction in lung tumor numbers in the *+/LSL-KrasG12D; Rb1*$^{\Delta L/\Delta L}$ mice. However, this reduction in lung tumors did not effect long-term survival of these mice. I further showed that DMBA, which can induce oncogenic ras mutations in mice, do not alter the tumor spectrum or affect tumor free survival of mice carrying the *Rb1*$^{\Delta L}$ mutation. These tumor studies show that loss of chromatin regulation by the LXCXE binding cleft of pRB does not universally enhance tumorigenesis. My work also shows that loss of chromatin regulation by pRB can inhibit tumorigenesis under some circumstances.

Over all, this thesis enhances our current understanding of the unique role of pRB among the pocket proteins in cell cycle regulation by showing how pRB utilizes LXCXE binding cleft mediated interactions to stably block the cell cycle, specifically in response to oncogenic stress signals. The pRB tumor suppressor pathway is inactivated in most cancers. Among pocket proteins, pRB is uniquely targeted for inactivation by mutations in a number of cancers. Investigating the molecular basis of this unique role for pRB in tumor suppression further can lead us to uncover novel tumor suppressive mechanisms that are important during cancer pathogenesis.

5.2 pRB regulation of a stable senescence arrest

My work, along with recent literature in the field, have highlighted a critical role for pRB in orchestrating a stable cell cycle arrest in response to the expression of activated oncogenes. Based on these studies our current understanding of pRB function during senescence can be summarized as follows (Fig. 5.1).

Expression of activated oncogenes such as HrasV12 induces replication stress resulting in the activation of DNA damage response signaling (DDR) (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). This is characterized by the expression of cyclin dependent kinase inhibitors (CKIs) such as p21 and p16 that inhibit Cyclin/CDK complexes that are responsible for phosphorylation and inactivation of pRB. The hypo-phosphorylated pRB directly binds to and inhibits the E2F transcription factors. This is essential for the transcriptional repression of key genes involved in DNA replication. pRB is indeed found to be enriched at these E2F target gene promoters during senescence and acute knock down of pRB results in de-repression of these genes and deregulated DNA synthesis during senescence (Chapter 2 and (Chicas et al., 2010)). Thus, in response to oncogenic stress pRB regulates cell cycle exit and entry into senescence by direct repression of E2Fs and E2F dependent transcription. The $Rb1^{\Delta L/\Delta L}$ fibroblasts in which the pRB-E2F interactions are intact exit the cell cycle and enter into senescence normally in response to oncogene expression (Chapter 2). This further emphasizes that acute cell cycle exit during senescence is primarily dependent on direct pRB-E2F interactions (Fig 5.1 top).

However, the $Rb1^{\Delta L/\Delta L}$ mutant cells ultimately re-enter the cell cycle and can resume proliferation indicating that later pRB dependent steps in establishing a senescent arrest are critical for the stability of senescence (Chapter 2). Recent reports in the literature alongside my work uncovered a critical role for chromatin regulation by pRB that potentially contributes to this stable senescence arrest. pRB interacts with H3K4 demethylases Jarid1a and Jarid1b and mediates the removal of activating methylation (H3K4me3) on E2F target gene promoters during senescence (Chicas et al., 2012). pRB is also required for the enrichment of repressive histone methylation (H3K9me3) at these promoters (Chicas et al., 2010; Narita et al., 2003). The addition of H3K9me3 is

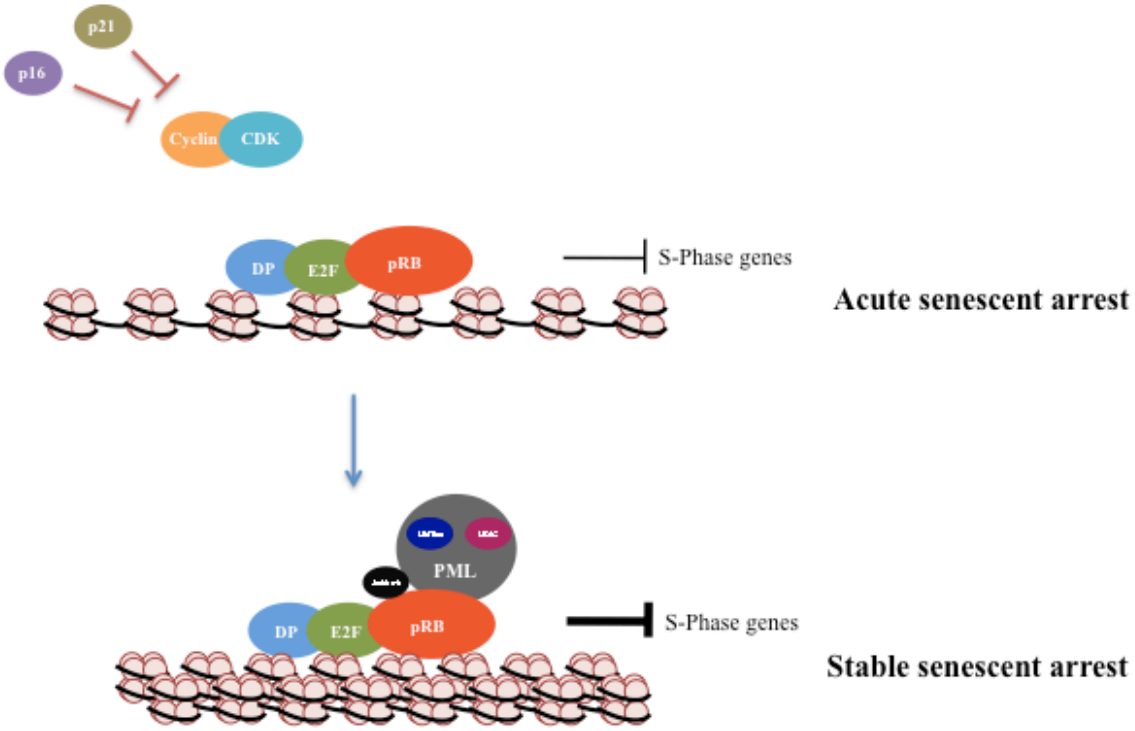

Figure 5.1 Proposed model of regulation of a stable senescence arrest by pRB

In response to activation by cyclin dependent kinase inhibitors (CKIs) such as p16 and p21 during senescence, pRB induces an acute cell cycle arrest by inhibiting cell cycle gene transcription by directly blocking trans-activation by E2Fs. Further, pRB cooperates with Promyelocytic leukemia protein (PML) to recruit chromatin regulating proteins that can assemble and spread heterochromatin, resulting in permanent silencing of proliferative genes.

defective in $Rb1^{\Delta L/\Delta L}$ fibroblasts implicating it in long term stability of senescence. The identity of histone methyl transferases responsible for the enrichment of this repressive mark during senescence is yet to be uncovered. Regardless of the precise mechanism of H3K9me3 deposition, these studies illustrated how pRB dependent chromatin regulation by means of removal of activating histone marks (H3K4me3) and enrichment of repressive modifications (H3K9me3) results in transcriptional repression across E2F responsive promoters. Further more, pRB mediates global compaction of chromatin and formation of senescence associated heterochromatic foci (SAHFs) thereby sustaining the stable repression of key cell cycle genes during senescence (Narita et al., 2003).

The exact signal that triggers the chromatin regulatory function of pRB is only beginning to be elucidated. Recent work including that presented in this thesis suggests that Promyelocytic leukemia (PML) bodies and pRB mediated recruitment of PML to the E2F target gene promoters might be at the core of the downstream chromatin changes during senescence (Chapter 3 and (Vernier et al., 2011)). PML bodies might serve as sites of nucleation of the proteins involved in chromatin assembly and aid in the heterochromatinization of cell cycle genes (5.1 bottom).

Since loss of pRB results in deregulated gene expression, DNA synthesis, and eventual escape from senescence, it is imperative that we further investigate the steps in gene silencing and higher order chromatin assembly that are controlled by pRB. In this way we will come to a thorough understanding of pRB function as a tumor suppressor protein.

5.3 Advantages of the gene targeted approach to study tumor suppressor protein function

Knockout mice have long been used to study tumor suppressor function of proteins (Ghebranious and Donehower, 1998). It has greatly aided in our understanding of the tumor suppressive ability of proteins like pRB and p53 both alone and in combination with other tumor prone models (Donehower, 1996; Lin et al., 1996). However, the knockout approach has some caveats while studying multifunctional proteins because it eliminates all of their functions. Complete loss of the protein prevents

the assignment of individual biochemical functions to the overall tumor suppressor ability of the protein. In addition, germ line deletion can also lead to compensation by related proteins thereby masking their normal physiological function. Gene targeted (Knock-in) mouse models circumvent some of these caveats in studying protein function (Kenzelmann Broz and Attardi, 2010; Taneja et al., 2011).

For example, pRB is a multifunctional protein capable of interacting with a number of other proteins and protein complexes to regulate the mammalian cell division cycle. Over a 100 cellular proteins have been reported that interact with pRB either directly or indirectly and the list continues to grow, emphasizing its functional complexity (Dick, 2007). Investigating the specific role of these different interactions in isolation and their relevance to tumor suppression is important for understanding pRB as a tumor suppressor protein. A structure-function approach, using point mutants of pRB, has helped separate some seemingly related functions. For example, a pRB mutant (Δ663) that is defective for E2F binding and cell cycle arrest was shown to still be capable of inducing markers of differentiation, suggesting they could be independent from one another (Sellers et al., 1998). However, *in vitro* overexpression studies can be misleading and the physiological relevance of these findings have to be validated using *in vivo* models. For example, *in vitro* experiments suggested that mutating the LXCXE binding cleft of pRB is sufficient to inhibit MyoD dependent terminal differentiation of cells into myotubes (Puri et al., 2001). However, gene targeted mice harboring mutations in the LXCXE binding cleft of pRB showed that these interactions are dispensable for muscle differentiation *in vivo* highlighting the advantages of gene targeted mouse models (Talluri et al., 2010).

Gene targeted models can serve as great tools to test and sometimes challenge accepted dogma about tumor suppressive functions of proteins that are largely based on knock out studies. This can lead to the discovery of novel functions that have been overlooked. A recent example of this is the $p53^{3KR/3KR}$ mice bearing lysine to arginine mutations at three p53 acetylation sites (Li et al., 2012). p53's role as a tumor suppressor has largely been attributed to its ability to induce cell cycle arrest, apoptosis or senescence in response to genotoxic stress. However, the $p53^{3KR/3KR}$ mice, despite being

defective for all the above functions still suppresses early onset spontaneous tumors in mice (Li et al., 2012). This model helped uncover a novel tumor suppressive role for unconventional activities of p53 in regulating energy metabolism and antioxidant function.

However, it is important to note that the advantages of gene-targeted approaches are limited by our understanding of the molecular determinants of individual protein-protein interactions. Most often, proteins use the same binding site to interact with multiple binding partners. For example, pRB has been show to interact with over 20 proteins through its LXCXE binding cleft (Dick, 2007). Consequently, attributing the *in vivo* phenotypes to specific interactions is still a challenge. In the future, co-crystal structures of proteins in functionally relevant complexes will be very useful in designing mutations that selectively disrupt interactions for making better gene targeted models.

5.4 Chromatin regulation during terminal differentiation

The normal development and terminal differentiation in $Rb1^{\Delta L/\Delta L}$ mice suggest that LXCXE interactions with pRB are dispensable for differentiation (Chapter 2). Furthermore, E2F target genes in differentiated muscle from the $Rb1^{\Delta L/\Delta L}$ mice show similar abundance of heterochromatin modifications as wild type controls and they are properly silenced.

On the surface, our results might seem to contradict earlier reports suggesting that pRB mediates heterochromatinization of E2F target genes during muscle differentiation (Blais et al., 2007; De Falco et al., 2006). This raises the question of what regulates heterochromatinization of cell cycle genes during terminal differentiation in $Rb1^{\Delta L/\Delta L}$ mice. Since $pRB^{\Delta L}$ is capable of binding to E2Fs normally, it is possible that the heterochromatinization of these genes during differentiation is an indirect consequence of cell cycle exit. During muscle differentiation, pRB acts by negatively regulating the E2Fs and also by positively affecting the MyoD dependent transcriptional program (De Falco et al., 2006). This might enable the repressive chromatin complexes to be recruited to cell cycle genes independently of pRB to modify chromatin and permanently silence them. Consistent with this interpretation, Andrusiak et al. recently demonstrated that pRB-E2F

regulation alone is sufficient to induce heterochromatin changes in terminally differentiated neurons (Andrusiak et al., 2013).

Alternately, p107 and p130 could have a compensatory role for enriching repressive chromatin marks during differentiation in the absence of the pRB-LXCXE interactions. Both p107 and p130 have been shown to have overlapping roles during development and differentiation (Classon and Dyson, 2001; Cobrinik et al., 1996). In addition, both these proteins have a LXCXE binding cleft and interact with chromatin regulatory proteins such as HDACs through this binding cleft. However, we did not notice any increase in the expression of p107 in the $Rb1^{\Delta L/\Delta L}$ muscle suggesting this possibility is less likely (Talluri et al., 2010). It will be interesting to test if chromatin regulation by the pocket protein family plays a direct role in heterochromatinization of the cell cycle genes during terminal differentiation. Generation of mice with multiple pocket protein family members defective for LXCXE interactions could answer some of these questions in a physiological setting.

5.5 pRB's unique role during cellular stress response

Among the pocket protein family pRB is selectively targeted for disruption by direct mutations in a number of cancers, most notably retinoblastoma and small cell lung cancer, suggesting a unique role for pRB in tumor suppression within its family (Sherr and McCormick, 2002). Disruption of pRB alone is also tumorigenic in mouse models whereas disruption of p107 and p130 is not (Classon and Harlow, 2002; Harrison et al., 1995). This raises the question of what the molecular basis of the unique tumor suppressive ability of pRB is.

This study along with other recent literature in the field provides important clues regarding the unique roles of pRB that are potentially tumor suppressive *in vivo*. Our work suggests that cellular stress stimuli that are potentially tumor promoting, activate the stress responsive pathways that uniquely engage pRB function. These stress stimuli include activation of oncogenes, genotoxic agents that induce DNA damage such as γ irradiation and reactive oxygen species (ROS).

My work along with others show that oncogenic stress uniquely activates a silencing program mediated by pRB to stably repress a group of cell cycle genes involved in DNA replication and S-Phase progression (Chicas et al., 2010; Talluri et al., 2010). In response to the expression of activated oncogenes pRB specifically binds to these gene promoters to assemble heterochromatin and stably silence their expression. This function seems to be unique to pRB and can't be compensated by the other pocket proteins p107 and p130. Other stimuli activate this unique growth inhibitory function of pRB as well. *Rb1*$^{\Delta L/\Delta L}$ fibroblasts are defective in arresting in response to DNA damage induced by γ-irradiation and expression of the CDK inhibitor p16^{INK4a} (Francis et al., 2009). Both activate the pRB pathway and induce senescence in MEFs (Harrington et al., 1998; Takahashi et al., 2006). Apart from MEFs, mammary epithelial cells and keratinocytes from the *Rb1*$^{\Delta L/\Delta L}$ mice are defective in arresting in response to TGF-β treatment, which is a potent growth inhibitor of these cell types (Francis et al., 2009). Interestingly, TGF-β is activated in response to irradiation and has been shown to play a role in response to genotoxic stresses (Barcellos-Hoff et al., 1994; Kirshner et al., 2006). Furthermore, oxidative stress and chronic TGF-β stimulation is known to induce senescence in certain cell types (Ksiazek et al., 2007; Yu et al., 2009). Collectively, these growth arrest signals that fail to arrest *Rb1*$^{\Delta L/\Delta L}$ cells indicate that the mutant cells have a unique deficit in responding to stress induced growth arrest signals.

5.6 Studying senescence in mouse fibroblasts in comparison with human

The cellular and molecular differences between senescence in mouse and human cells were detailed in the introduction to this thesis. The Arf-p53 pathway is considered to be the dominant pathway regulating senescence in mouse cells, compared to the p16/pRB pathway. This argument mainly stems from the observation that MEFs from *p16*^{Ink4a} null or *Rb1*$^{-/-}$ embryos enter at least a partial state of senescence in culture and in response to oncogenic stress (Krimpenfort et al., 2001; Sharpless et al., 2001; Sherr and DePinho, 2000). On the other hand, MEFs from *p19*^{Arf} *null* or *Trp53*$^{-/-}$ mice continue to proliferate under the same circumstances (Harvey et al., 1993; Kamijo et al., 1997). Consequently, most studies investigating the role of pRB during senescence use human

fibroblasts. In addition, mouse fibroblasts do not form senescence associated heterochromatin foci (SAHFs) similar to human diploid fibroblasts (HDFs) undergoing senescence (Itahana et al., 2004). As a result there are many gaps in our knowledge of chromatin changes in senescent mouse fibroblasts.

It is interesting to note that pRB acts downstream of the INK4/ARF locus that encodes for both the $p16^{Ink4a}$ and $p19^{Arf}$ proteins (Sherr, 2012). *Rb1*$^{-/-}$*/p107*$^{-/-}$ MEFs and *Rb1*$^{-/-}$*/p107*$^{-/-}$*/p130*$^{-/-}$ MEFs (TKO MEFs) fail to senescence in response to oncogenic ras despite normal induction of the p53 and p21 proteins suggesting that the pathway regulated by the pocket proteins is required for efficient senescence in MEFs (Peeper et al., 2001). Furthermore, acute depletion of pRB in MEFs is shown to be sufficient for escape from senescence and these escaped cells start synthesizing DNA and re-enter the cell cycle indicating that pRB is essential for stable maintenance of the senescence state in MEFs (Sage et al., 2003). Therefore, understanding the molecular mechanisms of the senescence arrest in mouse cells and the basis of the differences between mouse and human senescence is of great importance. This is particularly vital in order to interpret data from cells derived from knockout mice and for studying senescence and its contribution to tumor suppression *in vivo* using mouse models.

My study shows that the fundamental feature of senescence that is common between MEFs and human cells is the stable repression of E2F target cell cycle genes involved in DNA replication and S phase progression. I showed that LXCXE cleft mediated interactions with pRB are critical for this stable repression of E2F target cell cycle genes during senescence (Chapter 2). This function of pRB is crucial for the permanence of the senescence arrest as disruption of LXCXE cleft mediated interactions in our *Rb1*$^{\Delta L/\Delta L}$ MEFs allows escape from senescence and results in transformation. Importantly, this function is unique to pRB and cannot be compensated by the other pocket proteins p107 and p130. A recent study by Chicas et al. used IMR90 human diploid fibroblasts and has come to a similar conclusion by showing that pRB is recruited to these genes during oncogene induced senescence and is required for their stable repression (Chicas et al., 2010). Furthermore, by using shRNA's targeting pRB, p107 or p130 they showed that this function is unique to pRB. Taken together, these studies show

that RB's unique ability to silence key cell cycle genes during senescence is a critical component of the cellular response to oncogenic stress and is highly conserved across species.

Some of the differences observed during premature senescence in mouse vs. human cells are due to the oxygen sensitivity of MEFs in culture (Coppe et al., 2010). MEFs are highly sensitive to oxygen levels (20%) used in standard cell culture (Parrinello et al., 2003). The effect of oxidative stress is often one of the underappreciated aspects while studying premature senescence in MEFs. Reactive oxygen species (ROS) and oxidative DNA damage is one of the primary causes of mutations contributing to ageing and age associated diseases such as cancer (Denver et al., 2009; Kryston et al., 2011; Sedelnikova et al., 2010). Strikingly, MEFs grown under standard cell culture conditions (20% O_2) have about 3 fold higher mutation frequency compared to the cells cultured at physiological oxygen levels (3%) with an additional 3 fold increase in mutation rate upon spontaneous immortalization (Busuttil et al., 2003). Although oxidative stress induces premature senescence in MEFs, it might also contribute to the spontaneous escape from this senescence arrest as a result of high mutation rate. This makes it difficult to predict *in vivo* outcomes in cancer models based on the *in vitro* experiments done under these conditions. It is possible that oxidative stress might cooperate with or exacerbate some of the effects of $Rb1^{\Delta L}$ mutation thereby contributing to the increased spontaneous escape from oncogene induced senescence and transformation that we observe in $Rb1^{\Delta L/\Delta L}$ MEFs in culture. Less oxidative stress and spontaneous mutation rate under physiological conditions would allow additional tumor suppressor pathways such as p53 to delay and/or inhibit tumor progression *in vivo*. Tumor progression would require eventual loss of these additional checkpoints. Whether the $Rb1^{\Delta L/\Delta L}$ MEFs are more sensitive to oxidative stress in culture compared to the wild type cells and if they have increased mutation frequency has yet to be determined. More studies are needed to determine the effect of oxidative stress due to culture conditions and how it contributes to the differences in senescence phenotypes between mouse and human cells.

5.7 Chromatin assembly during senescence and its contribution to tumor suppression

Senescence is associated with a number of chromatin changes (Adams, 2007; Funayama and Ishikawa, 2007; Narita, 2007). However, the importance of each of these chromatin changes to the senescence phenotype is still not completely understood. Are they simply a consequence of senescence or do they actively contribute to the senescent state? Which of the chromatin changes associated with senescence are essential for induction and/or maintenance of senescence arrest *in vivo*? Recent reports in the literature have tried to address some of these issues.

The precise role of large scale chromatin compaction and its contribution to senescent arrest is still not clear. In some human diploid fibroblasts (HDFs) like IMR90 senescence is associated with condensation of whole chromosomes into structures called senescence associated heterochromatic foci (SAHFs) (Narita et al., 2003). However, SAHF formation is not a universal feature of senescence. It is specific to some cell types and certain senescence inducing signals suggesting that SAHFs are not a requirement for senescence (Kosar et al., 2011). Our work in mouse embryonic fibroblasts (MEFs) suggests that senescence in this cell type is associated with re-organization of heterochromatin without the condensation of whole chromosomes. This is evident by increased compaction of chromatin into fewer, but bigger heterochromatin bundles. Interestingly, chromatin compaction is defective in $Rb1^{\Delta L/\Delta L}$ MEFs suggesting that it is LXCXE dependent. Whether this is an indirect consequence of the inability of $Rb1^{\Delta L/\Delta L}$ MEFs to heterochromatinize E2F target genes or if it is an independent function of pRB has to be determined. A recent study showed that incorporation of repressive heterochromatin marks and SAHF formation are separable, suggesting that they could be independently regulated (Chandra et al., 2012). Nevertheless, large scale chromatin compaction might play a role in the permanence of senescence arrest by stable silencing of proliferative genes. Disruption of SAHFs in the cells that form them, have been shown to result in de-repression of cell cycle genes such as cyclin A and MCM3 (Narita et al., 2006).

The best *in vivo* evidence available so far for the role of heterochromatin modifications during senescence come from mice lacking Suv39h1, the histone methyl transferase capable of tri-methylating H3K9 (Braig et al., 2005). In response to oncogenic ras expression, splenocytes from the *Suv39h1*$^{-/-}$ mice fail to arrest and show defective accumulation of oncogene induced senescence markers such as H3K9me3, heterochromatin protein 1(HP1), and SA-β-gal. Furthermore, *Suv39h1*$^{-/-}$ mice expressing oncogenic NrasG12D in their hematopoietic compartment develop aggressive cancers and succumb to the disease significantly sooner than the control mice. This demonstrates that Suv39h1 mediated H3K9-trimethylation is important for senescence and tumor suppression *in vivo* (Braig et al., 2005). It is also possible that germ line deletion of Suv39h1 could have pleotropic effects that might affect tumorigenesis in this model. Conditional inactivation of Suv39h1 in the context of oncogene activation will be more informative in addressing the role of chromatin assembly specifically during oncogene induced senescence and tumorigenesis. More experiments are needed to determine if the increased tumor susceptibility is tissue specific, and future crosses of the *Suv39h1*$^{-/-}$ mice with other tumor prone models will tell us if loss of Suv39h1 has broader effects on senescence and tumor suppression *in vivo*. The data from our *Rb1*$^{\Delta L/\Delta L}$ mice, that have defects in the incorporation of the H3K9me3 repressive mark specifically during senescence suggests that tissue and cell type specificity, and the type of mutations may play a role in determining the course of cancer development *in vivo*.

5.8 The pRB LXCXE binding cleft in tumor suppression

Rb1$^{\Delta L/\Delta L}$ mice do not develop spontaneous tumors. In an interesting parallel, disruption of the *CDKN2A* gene which encodes for p16^{Ink4a} protein that acts upstream of pRB causes only a rare incidence of spontaneous tumors (Krimpenfort et al., 2001; Sharpless et al., 2001). However, these mice have increased susceptibility to tumorigenesis when crossed with a number of different cancer prone models. This suggests that the p16^{Ink4a} /pRB pathway might have context specific roles in tumor suppression in mouse models of cancer, or is activated in response to specific stress signals.

Interestingly, *Rb1*$^{\Delta L/\Delta L}$ mice show increased susceptibility to cancer in a *Trp53*$^{-/-}$ background (Coschi et al., 2010). Compound mutant mice have an altered tumor spectrum with an increased number of sarcomas and more aggressive tumors. Strikingly, these tumors show high levels of genomic instability compared to *TP53*$^{-/-}$ tumors. Furthermore, the *Rb1*$^{\Delta L}$ mutation promotes loss of heterozygosity and hastens tumor formation in *TP53*$^{+/-}$ mice (Coschi et al., 2010). This suggests that one of the key tumor suppressive roles of the pRB-LXCXE binding cleft is to maintain genome stability. In *Rb1*$^{\Delta L/\Delta L}$ mice the p53 pathway might be acting as a secondary barrier to eliminate genomically unstable cells thus preventing spontaneous tumor formation. In the absence of this secondary checkpoint in the *TP53*$^{-/-}$ background the genomically unstable cells in the *Rb1*$^{\Delta L/\Delta L}$ mice progress towards tumorigenesis unhindered (Manning et al., 2013).

Rb1$^{\Delta L/\Delta L}$ mice also cooperate with p53 loss during Wap-p53 (R172H) induced mammary tumors (Francis et al., 2011). However, the *Rb1*$^{\Delta L}$ mutation does not promote tumorigenesis in the Neu oncogene induced mammary tumor model or DMBA chemical carcinogen model (Francis et al., 2011)(Chapter 4 of this thesis). Interestingly, the same *Rb1*$^{\Delta L}$ mutation reduces lung tumors in response to the activation of oncogenic KrasG12D (Chapter 4). These results point to the need for more studies in order to understand the context specific role of the LXCXE binding cleft and how the *Rb1*$^{\Delta L}$ mutation affects tumorigenesis *in vivo*. Recent reports in the literature and unpublished work from our lab provide some clues in this regard.

The *Rb1*$^{\Delta L/\Delta L}$ MEFs show evidence of increased replication stress pointing to a role for pRB and the LXCXE cleft in maintaining genome integrity (C. Coschi, unpublished). The oncogenic ras-MAPK pathway induces replication stress causing DNA damage (Di Micco et al., 2006). Replication stress can promote tumorigenesis if the DNA damage is allowed to accumulate as a result of inactivation or loss of cellular DNA damage response pathways that limit the damage by inducing apoptosis and senescence(Halazonetis et al., 2008). However, the threshold of replication stress was shown to impact tumor progression *in vivo* with very high replication stress resulting in death of precancerous cells that are defective for stress response (Bartek et al., 2012; Murga et al., 2011). Therefore, it is tempting to speculate that activation of the

oncogenic ras pathway in combination with the *Rb1*$^{\Delta L}$ mutation might result in elevated replication stress that is too high to the cell to handle. This could potentially lead to the activation of the apoptosis pathway depending on the cell and tissue type, thus eliminating these precancerous cells. However, disruption of the DDR pathway by loss of proteins such as p53 allows these cells to survive and progress towards cancer.

Overall, these studies suggest that the tumor suppressor function of the pRB-LXCXE binding cleft could be highly context specific and crossing the *Rb1*$^{\Delta L/\Delta L}$ mice with different tumor prone models will help us better understand the signals that impinge on this function of pRB to suppress tumorigenesis.

5.9 Future perspectives

The role of PML during senescence is well documented (Bischof et al., 2002; Salomoni and Pandolfi, 2002). Our work in *Rb1*$^{\Delta L/\Delta L}$ MEFs suggests that pRB-PML interaction could act as a molecular switch that triggers heterochromatin assembly during senescence and determines the stability of senescence arrest. However, the signaling events that trigger this interaction are still not known. One of the potential signals is the posttranslational modification of the PML protein. There are multiple isoforms of the PML protein and PML is a target for extensive posttranslational modifications in response to diverse stress signals (Cheng and Kao, 2012; Jensen et al., 2001). However, the specific role of individual isoforms of PML and their posttranslational modifications during senescence is yet to be determined. Sumoylation is important for nuclear body formation and the function of PML (Seeler and Dejean, 2001). Interestingly, expression of SUMO-2/3 induces senescence in a p53 and pRB dependent manner (Li et al., 2006). This study suggests that Sumoylation could potentially act as a trigger that induces the interaction between PML and pRB during senescence. The presence of higher molecular weight bands of PML in our interaction assays also suggests this possibility. Future experiments aimed at identifying the senescence specific modifications of PML isoforms and pRB will enhance our mechanistic understanding of the signaling events that regulate pRB-PML interactions during senescence and their role in chromatin assembly.

The role of large scale chromatin compaction during senescence and its impact on the stability of the arrest is another key question. The *Rb1*$^{\Delta L/\Delta L}$ MEFs show defective chromatin compaction during senescence suggesting a role for pRB and the LXCXE binding cleft in this process. Knockdown of pRB in IMR90 human fibroblasts also disrupts the formation of SAHFs in these cells suggesting that pRB plays an important role in genome wide chromatin compaction (Chandra et al., 2012; Narita et al., 2003). It is still not clear if pRB has a direct role in this process or if it acts upstream to promote chromatin compaction. Disruption of already formed SAHFs by acute depletion of pRB in IMR90 cells suggests a direct role (Chandra et al., 2012; Narita et al., 2003). However more precisely timed experiments are needed to confirm this. Experiments done in *Rb1*$^{\Delta L/\Delta L}$ fibroblasts hint at a potential pRB interaction that could mediate such global compaction of chromatin during senescence. pRB interacts with the Condensin-II complex through its LXCXE binding cleft and this interaction is necessary for proper condensation of mitotic chromosomes (Coschi et al., 2010; Longworth et al., 2008). Interestingly, unlike Condensin-I complex, which needs nuclear envelope breakdown during prophase to bind to chromatin, the Condensin-II complex is present in the nucleus during interphase and can associate with chromatin throughout the cell cycle (Hirano, 2005). So, one intriguing possibility is that the pRB-Condensin-II complex co-operates to induce chromatin compaction and SAHF formation during senescence. Knocking down Condensin-II subunits in fibroblasts undergoing senescence will tell us if this is the case.

Much has been learned about senescence and the role of pRB in senescence over the last decade. But, there remain many unanswered questions. This thesis provides the groundwork for further exploring the role of the LXCXE cleft mediated chromatin regulation by pRB during senescence.

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Please print name: Fred Dick

Appendix C: List of Plasmids

Name	Genes encoded	Obtained/ Constructed	Resistance marker	Stock Number
pBABE	Puromycin	Dr. R. Hurford	Ampicillin Puromycin	28
pBABE-HrasV12	ras	Dr. M. Classon	Ampicillin Puromycin	450
pMOV-Psi	Ecotropic envelope glycoprotein	Dr. M. Classon	Ampicillin	530
pBABE-MyoD	MyoD	Srikanth Talluri	Ampicillin Puromycin	667
pscodon-GST-RB-LP	GST-RB-LP	Dr. Fred Dick	Ampicillin	526
pscodon-GST-RB-ΔL-LP	GST-RB-ΔL-LP	Srikanth Talluri	Ampicillin	668
pCIneo-FLAG-PML I	PML isoforms I	Dr. Lawrence Banks	Ampicillin	669
pCIneo-FLAG-PML II	PML isoforms II	Dr. Lawrence Banks	Ampicillin	670
pCIneo-FLAG-PML III	PML isoforms III	Dr. Lawrence Banks	Ampicillin	671
pCIneo-FLAG-PML IV	PML isoforms IV	Dr. Lawrence Banks	Ampicillin	672
pCIneo-FLAG-PML V	PML isoforms V	Dr. Lawrence Banks	Ampicillin	673
pCIneo-FLAG-PML VI	PML isoforms VI	Dr. Lawrence Banks	Ampicillin	674
pBABE-FLAG-PML-I	PML isoforms I	Srikanth Talluri	Ampicillin Puromycin	675
pBABE-FLAG-PML-II	PML isoforms II	Srikanth Talluri	Ampicillin Puromycin	676

pBABE-FLAG-PML-III	PML isoforms III	Srikanth Talluri	Ampicillin Puromycin	677
pBABE-FLAG-PML-IV	PML isoforms IV	Srikanth Talluri	Ampicillin Puromycin	678
pBABE-FLAG-PML-V	PML isoforms V	Srikanth Talluri	Ampicillin Puromycin	679
pBABE-FLAG-PML-VI	PML isoforms VI	Srikanth Talluri	Ampicillin Puromycin	680

Appendix D: List of Adenoviruses

Name	Genes encoded	Obtained/ Constructed
Ad-E2F1	E2F1	Dr. Erik Knudsen
Ad-GFP	GFP	Dr. Trevor Shepherd
Ad-Cre	Cre recombinase	Dr. Trevor Shepherd

Appendix E: List of Antibodies

Antibody name	Protein recognized	Species	Supplier	CAT. #	Application
E2F1 (KH95)	E2F1	Mouse IgG	Santa Cruz Biotechnology	SC-251	WB (1:500)
p107 (C-18)	p107	Rabbit IgG	Santa Cruz Biotechnology	SC-318	WB (1:500)
PCNA (PC10)	PCNA	Mouse IgG	Santa Cruz Biotechnology	SC-56	WB (1:500)
p130 (C-20)	p130	Rabbit IgG	Santa Cruz Biotechnology	SC-317	WB (1:500)
MCM7 (141.2)	MCM7	Mouse IgG	Santa Cruz Biotechnology	SC-9966	WB (1:500)
β-Actin	Actin	Rabbit IgG	Sigma	A2066	WB (1:500)
Lamin A/C	Lamin	Rabbit IgG	Chemicon	MAB3211	WB (1:1000)
BrdU (B44)	BrdU	Mouse IgG	BD biosciences	347580	IF (1:500), FC (1:200)
MHC (MF20)	Myosin heavy chain	Mouse IgG	Developmental studies Hybridoma bank, Univ. of Iowa	MF-20	IF (1:200)

H3K9me3	H3K9me3	Rabbit IgG	Millipore	07-442	IF (1:1000), ChIP (4µg)
H3K27me3	H3K27me3	Rabbit IgG	Millipore	07-449	IF (1:1000), ChIP (4µg)
Anti-PML clone 36.1-104 (monoclonal)	Mouse PML	Mouse ascites	Millipore	MAB3738	IF (1:300), WB (1:500), ChIP (4µg)
Anti-PML H-238 (polyclonal)	Multiple PML isoforms	Rabbit	Santa Cruz Biotechnology	(SC-5621)	WB (1:500)
Anti-phospho-Histone H2A.X (ser139) clone JBW301	Phospho-histone H2A.X	Mouse IgG	Millipore	05-636	IF (1:300), WB (1:500)
Anti-FLAG M2 antibody	FLAG peptide	Mouse IgG	Sigma	F-1804	IF (1:500), WB (1:1000), ChIP (4µg)
Anti Cyclin E	Cyclin E	Rabbit	Abcam	Ab7969	WB (1:500)
Anti-pRB (M153)	pRB	Rabbit	Santa Cruz Biotechnology	SC-7905	WB (1:500), ChIP (5µg)
Anti-p53-ser15	Phopho-p53 (ser15)	Rabbit	Cell signaling	#9284	WB (1:1000)
Ant-p21 (Ab-4)	p21	Mouse	Calbiochem	OP76	WB (1:500)
Anti-Pan-ras	ras	Rabbit	Santa Cruz Biotechnology	FL-189	WB (1:250)

Appendix F: PCR conditions for genotyping

PCR Conditions: Rb1-ΔLXCXE

Master Mix per reaction:

0.5 μL $MgCl_2$ (50mM stock)

2 μL dNTPs (2mM stock)

2 μL PCR Buffer (10X stock)

0.25 μL of FD-134 primer (20 μM stock)

0.25 μL of FD-135 primer (20 μM stock)

12.5 μL water

0.5 μL Taq

2 μL DNA

Reaction Conditions: Program SL01

94^{O}C for 2:30

94^{O}C for 0:20

60^{O}C for 0:20

70^{O}C for 2:00

Go to step #2, 29 times

72^{O}C for 10:00

12^{O}C until stopped

Expected Results:

Wild type band 136 bp

Mutant band 274 bp

Primers:

FD134: 5’ AGC TTC ATA CAG ATA GTT GGG 3’

FD135: 5’ CAC AAA TCC CCA TAC CTA TG 3’

PCR Conditions: KrasG12D

Master Mix per reaction:

0.6 µL $MgCl_2$ (50mM stock)

2 µL dNTPs (2mM stock)

2 µL PCR Buffer (10X stock)

0.5 µL of K1 primer (20 µM stock)

0.5 µL of K2 primer (20 µM stock)

0.5 µL of K3 primer (20 µM stock)

11.4 µL water

0.5 µL Taq

2 µL DNA

Reaction Conditions: Program RasG12D

95^{o}C for 2:00

95^{o}C for 0:30

61^{o}C for 0:30

72^{o}C for 0:45

Go to step #2, 34 times

72^{o}C for 10:00

4^{o}C until stopped

Expected Results:

Wild type band =~622 bp

LSL cassette=500 bp

1Lox (Recombined after Cre)=~650bp

Primer K1: 5’ GTC TTT CCC CAG CAC AGT GC 3’

Primer K2: 5’ CTC TTG CCT ACG CCA CCA GCT C 3’

Primer K3: 5’ AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A 3’

Appendix G: Primers and real time PCR conditions for ChIP

Mcm3 Forward Primer: 5' GAATGCAGTGCTTCCTAGCC 3'

Mcm3 Reverse Primer: 5' CGGAAGTTTATGGTGGAGGA 3'

Expected band size: 205bp

Ccne1 Forward primer: 5' GAGAACTTGGTAGACCAACTCTAAA 3'

Ccne1 Reverse primer: 5' GCAGCTGTTCTTAACTCTGTCTAGT 3'

Expected band size: 71bp

Master Mix per reaction:
5 μL iQSYBRGreen master mix (2X stock)
1.0 μL of Forward primer (10 μM stock)
1.0 μL of Reverse primer (10 μM stock)
1.0 μL RNAase /DNAase free water
2 μL ChIP DNA

Real time PCR conditions:

95^{o}C for 3:00
95^{o}C for 0:10
58^{o}C for 0:30
Go to step #2, 39 more times
95^{o}C for 0:10
Melt curve 65^{o}C to 95^{o}C increment 0.5 ^{o}C for 0:05
4^{o}C

Curriculum Vitae

Srikanth Talluri

Education:

PhD 2013
Dept. of Biochemistry
University of Western Ontario
Supervisor: Dr. Fred Dick

MSc (with distinction) 2002
Dept. of Biochemistry
Andhra University, Visakhapatnam, India

BSc 2000
Microbiology, Biochemistry and Applied nutrition
Nagarjuna University, Guntur, India

Research and professional experience:

- Graduate student in the laboratory of Dr. Fred Dick (2006-Present)
- Research assistant; Anticancer Drug discovery program, Dr. Reddy's Laboratories Ltd. Hyderabad, India (2002-2006)

Awards & Scholarships:

- Experimental oncology travel award (2012)
- Western Graduate Thesis Research award (2011)
- Experimental oncology travel award (2011)
- CIHR-STP (CaRTT) PhD studentship (2010-2011)
- Award for Best poster presentation (Cancer): "Epigenetics, Eh!" First Canadian conference on Epigenetics, London, ON (2011)
- Western Graduate Research Scholarship (2006-2011)

Publications:

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Matthew J. Cecchini, Daniel T. Passos, Michael Thwaites, **Srikanth Talluri**, Jean Leon Chong, Paul Cantalupo, M. Teresa Sáenz-Robles, Sarah M. Francis, Paul Stafford,

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Oral/Poster presentations (presenting author underlined):

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Srikanth Talluri, Alison L. Martens, and Frederick A. Dick. The Retinoblastoma protein's LXCXE binding cleft is important for the maintenance of a senescence arrest. DNA tumor virus meeting 2011, Trieste, Italy (Oral Presentation)

Srikanth Talluri, Christian E. Isaac, Alison L. Martens and Frederick A. Dick. Role of chromatin regulation in retinoblastoma protein mediated tumor suppression. Epigenetics, Eh! First Canadian conference on Epigenetics, May 4-7, 2011, London, ON

Talluri, S., Isaac, C.E., Ahmad, M., Henley, S.A., Francis, S.M., Martens, A.L., Bremner, R., and Dick, F.A. (2009) A G1 checkpoint mediated by the retinoblastoma protein that is dispensable in terminal differentiation but essential for senescence. First International Retinoblastoma Meeting, Toronto, Ontario, November 2009. (Oral presentation)

Talluri, S., Isaac, C.E., Martens, A.L., and Dick, F.A. (2008). The LXCXE binding cleft mediates a checkpoint function of the Retinoblastoma protein during cellular senescence. Mechanisms and Models of Cancer, Cold Spring Harbor, NY, August 2008.

Talluri, S., Isaac, C.E., Francis, S.M., Martens, A., and Dick, F.A. (2008). The Retinoblastoma Protein uses LXCXE type Interactions for a Stress Responsive G1 Arrest. Cell Cycle, Cold Spring Harbor, NY, May 2008. (Oral presentation)

Talluri, S., Isaac, C.E., Martens, A., and Dick, F.A. (2008). The Retinoblastoma Protein uses LXCXE type Interactions for a Stress Responsive G1 Arrest. Signaling in Normal and Cancer Cells. Banff, Alberta, March 2008.

Isaac, C.E., Francis, S.M., **Talluri, S**. and Dick, F.A. (2007). pRB uses LXCXE Interactions to Establish a Senescent Cell Cycle Arrest as part of its Tumor Suppressive Function. Molecular Biology of Small DNA Tumour Viruses, Trieste, Italy, July 2007. (Oral presentation)