Genetic analyses of the *Rb* and *p53* genes in murine development and tumorigenesis

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ABSTRACT

The *RB* and *p53* genes play central roles in cell cycle and checkpoint control, apoptosis and many other biological processes. These are central tumor suppressor genes that are each inactivated in many human cancers. Mouse genetic analyses have been used to dissect roles of these genes in development and cancer.

Targeted disruption of the retinoblastoma gene in mice leads to embryonic lethality in midgestation accompanied by defective erythropoiesis. *Rb*-/- embryos also exhibit inappropriate cell cycle activity and apoptosis in the central nervous system (CNS), peripheral nervous system (PNS), and ocular lens. Loss of *p53* can prevent the apoptosis in the CNS and lens; however, the specific signals leading to p53 activation have not been determined.

We tested the hypothesis that hypoxia caused by defective erythropoiesis in *Rb*-null embryos contributes to p53-dependent apoptosis and found upregulation of hypoxia-inducible genes in CNS tissue from *Rb*-/- embryos. The Cre-loxP system was used to generate embryos in which Rb was deleted in the CNS, PNS and lens, in the presence of normal erythropoiesis. In contrast to the massive CNS apoptosis in *Rb*-null embryos at E13.5, conditional mutants did not have elevated apoptosis in this tissue. There was still significant apoptosis in the PNS and lens, however. *Rb*-/- cells in the CNS, PNS, and lens underwent inappropriate S-phase entry in the conditional mutants at E13.5. By E18.5, conditional mutants had increased brain size and weight as well as defects in skeletal muscle development. These data supported a model in which hypoxia is a necessary cofactor in the death of CNS neurons in the developing *Rb*-mutant embryo.

Cells in the human retina are extremely sensitive to loss of function of the retinoblastoma tumor suppressor gene *RB*. Although retinoblastoma is thought to have developmental origins, the function of *Rb* in retinal development has not been fully characterized. We also used the Cre-loxP system to study the role of *Rb* in normal retinal development and in retinoblastoma in the mouse. In late embryogenesis, *Rb*-deficient retinas exhibited ectopic S-phase and high levels of p53-independent apoptosis, particularly in the differentiating retinal ganglion cell layer. During postnatal retinal development, loss of

Rb led to more widespread retinal apoptosis, and adults showed loss of rod photoreceptors and bipolar cells. Conditional *Rb* mutation in the retina did not result in retinoblastoma formation even in a *p53*-mutant background. We show that loss of *Rb* and the *Rb* family member *p130*-deficient leads to retinoblastoma.

Phosphorylation of the p53 tumor suppressor at Ser20 (murine Ser23) has been proposed to be critical for disrupting p53 interaction with its negative regulator, MDM2, and allowing p53 stabilization. To determine the importance of Ser23 for the function of p53 in vivo, we generated a mouse in which the endogenous p53 locus was targeted to replace Ser23 with alanine. We show that in mouse embryonic fibroblasts generated from Ser23 mutant mice, Ser23 mutation did not dramatically reduce ionizing radiation (IR)-induced p53 protein stabilization or p53-dependent cell cycle arrest. However, in Ser23 mutant thymocytes and in the developing cerebellum, p53 stabilization following IR was decreased and resistance to apoptosis was observed. Homozygous Ser23 mutant animals had a reduced lifespan, but did not develop thymic lymphomas or sarcomas that are characteristic of p53-/- mice. Instead, Ser23 mutant animals died between 1 and 2 years with tumors that were most commonly of B-cell lineage. These data support an important role for Ser20/23 phosphorylation in p53 stabilization, apoptosis and tumor suppression.

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Chapter 1: Introduction

Cancer is a genetic disease in which cellular proliferation has gone awry, as disruption of the cell cycle machinery uncouples proliferation from normal environmental control. Developing tumors must evade checkpoints that act to protect against tumor formation by inducing responses such as cell death (apoptosis). In addition, many other changes in cell physiology are important for tumor development and progression (Hanahan and Weinberg, 2000). The means through which cancer cells subvert normal cellular processes is largely through gene mutations, including the activation of proto-oncogenes and inactivation of tumor suppressor genes. This thesis describes the use of mouse genetics to elucidate *in vivo* functions of two central tumor suppressor genes, *Rb* and *p53*. These tumor suppressor genes encode proteins with broad, critical roles in protecting against tumorigenesis. The *Rb* and *p53* genes regulate checkpoint and proliferation control and also impact on many other biological processes. The main focus of this thesis is on the role of *Rb* in development and tumorigenesis, with particular emphasis on *Rb* function in the retina and in suppression of retinoblastoma. In addition, this thesis addresses a specific question regarding the regulation of p53 function, namely the importance of a p53 phosphorylation site for p53 activity.

Part A: The Retinoblastoma gene, *RB*

Retinoblastoma and Rb gene discovery

Studies of the rare pediatric eye tumor, retinoblastoma, have led to important insights into the nature of cancer. Examination of the pattern of inheritance of retinoblastomas eventually led to the very concept of a tumor suppressor gene as a gene that required

biallelic inactivation to promote tumor formation (Weinberg, 1991). Retinoblastoma patients present in two forms; in some patients, tumors are bilateral and multifocal, while in others, tumors are unilateral. Bilateral retinoblastomas have long been observed to often be familial (Smith and Sorsby, 1958). Statistical analyses of the pattern of inheritance of this tumor led to the hypothesis by Alfred Knudson that two hits are required for retinoblastoma; in one case, a mutation is passed along the germline, and only one more mutation is needed to unleash tumor formation (Knudson, 1971). Sporadic cases were unilateral as two independent mutational events were proposed to be needed.

Based on its mutation in retinoblastoma, the retinoblastoma susceptibility (*RB*) gene was the first tumor suppressor gene cloned. The discovery of the *RB* gene was rooted in early cytogenetic experiments. Chromosomal banding was used to show deletions in 13q14 in retinoblastomas, implicating a gene in this region as being responsible (Knudson et al., 1976). Restriction fragment length polymorphisms on normal and tumor DNA from both hereditary and sporadic retinoblastoma demonstrated that both forms of retinoblastoma lost the same chromosomal region and narrowed the region down (Cavenee et al., 1983). Furthermore, these studies provided mechanistic insight into allelic loss, demonstrating that mitotic recombination or chromosomal nondisjunction could lead to loss of the remaining wild-type allele (Cavenee et al., 1983). Later studies pinpointed the region of loss, and the retinoblastoma susceptibility locus was cloned (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Reintroduction of *RB* into *RB*-deficient tumor cell lines could revert neoplastic phenotypes, supporting a critical role for *RB* loss in tumorigenesis (Huang et al., 1988).

RB mutation in cancer

While there is exquisite sensitivity of the retina to tumorigenesis upon *RB* inactivation, other tissues in germline *RB* heterozygous humans are also sensitized to tumorigenesis (Eng et al., 1993). Early use of external beam radiation treatment for retinoblastoma exacerbated the development of secondary tumors in germline mutant *RB* patients, in particular, osteosarcoma, and soft-tissue sarcomas, presumably by increasing the rate of mutations (Eng et al, 1993). Secondary tumor incidence is also increased in germline *RB*+/- patients even without radiotherapy (Eng et al., 1993; Fletcher et al., 2004). Analyses of the *RB* gene has led to the finding that *RB* is mutated widely in human cancers, including osteosarcomas and various soft tissue sarcomas, as well as small-cell-lung, breast, brain, prostate and other sporadic cancers (Bookstein et al., 1990; Eng et al., 1993; Harbour et al., 1988; Horowitz et al., 1990; Stratton et al., 1989; Venter et al., 1991). The spectrum of tissue types in which *RB* mutations are found suggests that *RB* is critical for suppressing cancer in many tissues.

pRB function in the cell cycle

The gene responsible for retinoblastoma has since been found to be a central node in regulation of proliferation, and virtually all cancers feed through the *RB* pathway. The *RB* gene contains 27 exons found over about 180 kB and encodes a 4.7kB mRNA product (Bookstein et al., 1988; Hong et al., 1989). The protein product of this gene, pRB is a 110kDa phosphoprotein. Insight into the function of the *RB* gene came from findings that pRB binds to DNA tumor viral proteins such as Adenovirus E1A (Whyte et al.,

1988), HPV-16 E7 (Dyson et al., 1989b) and SV40 Large T antigen (DeCaprio et al., 1988; Ewen et al., 1989). Interestingly, these DNA viral oncoproteins targeted only the hypophosphorylated form of pRB. The region of viral oncoproteins that bound pRB were essential for the transforming ability of the virus, suggesting that binding to pRB is important for viral transformation (Whyte et al., 1988; Whyte et al., 1989).

Early studies critically noted that pRB in the normal cell cycle is regulated by phosphorylation. pRB is generally found highly phosphorylated in proliferating cells, and dephosphorylated in differentiating cells (Chen et al., 1989). Hypophosphorylated pRB was shown to exist in a complex with E2F, which was originally identified as a cellular factor required for adenoviral E1A to activate adenoviral E2 expression (Kovesdi et al., 1986). pRB/E2F complexes were most easily detected in G1 phase of the cell cycle. This complex could be disrupted by the introduction of the adenovirus E1A protein, suggesting that pRB may function by inhibiting E2F transcription factor activity, and E1A may act to inactivate pRB to promote proliferation (Bandara and La Thangue, 1991; Chellappan et al., 1991). cDNAs encoding a gene with E2F properties (now known as *E2F1*) that bound pRB were soon identified (Helin et al., 1992; Kaelin et al., 1992). *E2F1* was the first of multiple related family members to be identified and now known to be important for the biological activity of pRB.

The phosphorylation of pRB as cells as cells approach the G1/S transition is reminiscent of the decision point defined by Pardee as the 'restriction point'. This is a point during G1 in which mitogenic stimulation is no longer required for cell cycle progression, and

commitment towards S-phase entry occurs (Pardee, 1974). Introduction of pRB protein into cells through microinjection in early G1 could lead to inhibition of progression into S-phase, suggesting that pRB functioned as a regulator of the cell cycle in G1 (Goodrich et al., 1991). Mouse embryonic fibroblasts (MEFs) derived from *Rb-/-* embryos exhibited a shortened G1 phase, consistent with a role for pRB in G1/S transition (Herrera et al., 1996). pRB may also have broader functions in the cell cycle, including in S phase. A phosphorylation-site-mutant form of pRB when ectopically expressed was able to inhibit cells in S-phase (Knudsen et al., 1998). pRB is maintained phosphorylayed through the late stages of the cell cycle and dephosphorylated in M-phase (Ludlow et al., 1993).

pRB family

pRB is now known as one member of a family of proteins, the so called "pocket protein family" that includes two other members, p107 and p130. These proteins have their highest similarity in this pocket region (Ewen et al, 1991, Dyson et al, 1992). The pRB pocket contains an A and B domain (separated by a spacer) that is critical for viral oncoprotein binding, and mutations in *RB* found in retinoblastoma tumors often affect the pocket region structure (Harbour, 1998). It is the pocket region that binds and inhibits E2F family members. The pocket region also binds to DNA tumor viruses, including adenovirus E1A, high-risk papillomavirus E7 and SV40 Large T antigen. These viral proteins all bind pRB via a critical LxCxE motif (Dyson et al., 1989b; Whyte et al., 1988). The crystal structure of a LxCxE containing peptide from E7 shows this domain binds the B-box portion of pRB, and that the residues critical for binding are highly conserved in p107 and p130 (Lee et al., 1998).

p107 was identified as a cellular protein that bound to E1A or SV40 Large T Ag in regions needed for transformation (Dyson et al., 1989a; Whyte et al., 1989). p130 was similarly identified as binding a similar region of E1A (Dyson et al., 1992). Outside of the pocket region, p107 and p130 are more highly similar to each other compared to pRB (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). p107 and p130 have a spacer region, lacking in pRB, between the A and B pockets that mediates the formation of stable complexes with cyclin/CDK2, critical regulators of pRB family phosphorylation (Ewen et al., 1992; Hannon et al., 1993; Lees et al., 1992). This stable binding is not found with pRB. The function and importance of formation of these stable complexes is not clear, although they may play a role in inhibition of CDK2 activity (Castano et al., 1998; Lacy and Whyte, 1997). The pocket proteins differ in their pattern of expression through the cell cycle and preferences for binding to E2F family members (Trimarchi and Lees, 2002). p130 is generally expressed in cells that have exited the cell cycle, while p107 expression increases as cells proceed towards S-phase (Classon and Harlow, 2002; Richon et al., 1997). p107 is generally expressed at low levels in differentiated cells (Classon and Harlow, 2002; Richon et al., 1997). The fact that DNA tumor viral oncoproteins have evolved to target all three pocket proteins raises the possibility that inhibition of p107 and p130 activity may also be tumorigenic. However, while Rb is widely mutated in human cancers, there are rare instances of documented *p130* or *p107* mutations. *p130* was found mutated in a small-cell lung cancer cell-line (Helin et al., 1997) and, in a heterozygous state, in nasopharyngeal carcinoma tumor samples (Claudio et al., 2000). While the pocket protein genes are similar structurally, it is not known why *RB* specifically is widely mutated in cancer.

Upstream pRB regulation: CDKs

Different complexes of cyclin and cyclin dependent kinases (CDKs) phosphorylate pRB in different stages of the cell cycle. Mitogenic stimulation causes upregulation of D-type cyclins (D1, D2, D3) that form a holoenzyme with either CDK4 or CDK6 to initially phosphorylate pRB. Thus, extracellular signals are coupled to the decision enter the cell cycle. D-type cyclin transcription, translation protein stability and assembly into active complexes are all important for activation and are all regulated by mitogenic signaling (Sherr and Roberts, 1999). pRB is then further phosphorylated by CDK2 in association with cyclin E (E1 and E2) late in G1. Cyclin E is a direct target of the pRB/E2F pathway, which controls pRB phosphorylation in a positive regulatory loop. Phosphorylation of pRB by cyclin E/CDK2 appears to require initial phosphorylation by cyclin D/CDK4/6 (Lundberg and Weinberg, 1998). The importance of pRB family regulation is seen in the frequent activation of Cyclin D in human cancers. Many cancers have upregulated cyclin D expression, which is not surprising as cyclin D1 is a target of many oncogenic pathways. In addition, direct amplification of the *cyclin D1* locus has been found in breast cancers, and translocation of *cyclin D1* to the *IgH* locus has been found in B cell lineage tumors such as multiple myeloma (Bergsagel and Kuehl, 2003). These data are strongly supportive of a causal role for cyclin D overexpression in tumorigenesis. *CDK4* is a frequent target of amplification in glioblastomas (Schmidt et al., 1994), and, in melanoma kindreds, activating *CDK4* mutations have been found making the mutant protein insensitive to inhibition by the CDK inhibitor (CDKI) p16 (Zuo et al., 1996).

Thus, in addition to direct *RB* gene inactivation in cancer, upstream regulators of pRB function are also clear targets in tumorigenesis.

Upstream pRB regulation: CDKIs

CDKs are inhibited by two classes of cyclin dependent kinase inhibitors (CDKIs). The first class, the Cip/Kip family, includes p21Cip1, p27Kip1 and p57Kip2. These proteins bind both the cyclin and CDK partners, affecting the activity of cyclin D-, cyclin A- and cyclin E-dependent kinases (Sherr and Roberts, 1999). It was originally thought based on in vitro experiments that Cip/Kip class CDKIs inhibit both CDK4/6 and CDK2. However, more recent work suggests that these inhibitors may actually be positive regulators of CDK4/6-cyclin assembly and activation, and function mainly as inhibitors of CDK2. Consistent with this, cells from mice lacking p21 and p27 were strongly impaired in assembly of CyclinD/CDK complexes (Cheng et al., 1999). Also, p21 and p27 promoted the activity of cyclinD/CDK complexes, and accumulation in the nucleus (LaBaer et al., 1997). CyclinD/CDK4 appears to sequester p21 and p27 away from inhibiting CDK2 (Polyak et al., 1994; Reynisdottir et al., 1995). p27 may play a role in tumor suppression. *p27* heterozygous mice challenged with a carcinogen or irradiation were prone to tumors, while the remaining wild-type allele was retained and expressed (Fero et al., 1998). This illustrates that gene dosage decreases can contribute to tumorigenesis even when partial gene function is intact.

The second class of CDKIs are proteins of the INK4 (INhibitors of CDK4) family, including p16INK4a, p15INK4b, p18INK4c and p19INK4d (Sherr and Roberts, 1999).

These bind only to CDK4 and CDK6, and not to the cyclin partner. A model for INK4 inhibition of the cell cycle is through displacing members of the CIP/KIP family from CDK4/6, thus allowing CIP/KIP inhibitors to inhibit CDK2. Members of the INK4 members are regulated differently. p15INK4b is induced by TGFbeta and may be important for the inhibitory effects of TGFbeta on proliferation in some cells (Hannon and Beach, 1994). Of the INK4 members, p16INK4a has been clearly demonstrated to function as a tumor suppressor. *p16INK4a* is frequently mutated in melanoma, pancreatic carcinoma and other human cancers (Liggett and Sidransky, 1998). The *p16INK4a* gene is found in an area of frequent chromosomal loss, 9p21. The remaining wild-type copy of *p16INK4a* is sometimes deleted, but is often inactivated by promoter methylation (Little and Wainwright, 1995). Thus, inactivation of a tumor suppressor gene can occur through genetic or through epigenetic means. Intriguingly, the *INK4a* locus also encodes a second gene that uses an alternative 1st exon, (exon 1 beta) and encodes a protein unrelated to p16 in amino acid sequence(Quelle et al., 1995). Remarkably, the product of this transcript, p14ARF (p19ARF in mice), is also a tumor suppressor, that regulates the p53 pathway(Zhang et al., 1998) (as discussed in section C). Inactivation of either *p14ARF* or *p16INK4a* in mice leads to tumor formation, supporting the idea that each has tumor suppressor function (Kamijo et al., 1999; Serrano et al., 1996; Sharpless et al., 2001).

pRB and E2F effectors

Major downstream mediators of the effects of pRB on the cell cycle are E2F transcription factors. E2F family members function as heterodimeric transcription factors, binding to a

partner protein DP1 or DP2 (Trimarchi and Lees, 2002). pRB binds to the transactivation domain of E2F, inhibiting E2F transactivation (Flemington et al., 1993; Helin et al., 1993; Hiebert et al., 1992; Weintraub et al., 1992). pRB also acts as an active repressor, recruiting repressive complexes to E2F sites to actively inhibit transcription (Trimarchi and Lees, 2002). Phosphorylation of pRB is thought to lead to both a loss of active repression as well as an ability of free E2F to function as a positively acting transcription factor.

To date, eight E2Fs have been cloned; these have been classified into different groups. The best characterized of there are E2Fs 1-5 (Trimarchi and Lees, 2002). E2F1,2,3a are known as the 'activating E2Fs'. Expression of E2F1-3 could drive quiescent cells into the cell cycle (Johnson et al., 1993; Lukas et al., 1996). These have an N-terminal nuclear localization signal (NLS) and interact with pRB. The *E2F3* gene encodes E2F3a and E2F3b, which have different promoters and different 5' coding exons (Leone et al., 2000). E2F3b has been implicated in repressive E2F function, however, this has not been extensively characterized. E2F4 and E2F5 are thought to function largely as repressive E2Fs; ectopic expression of these cannot drive quiescent cells into the cell cycle (Muller et al., 1997; Verona et al., 1997). E2F4 and 5 bind predominantly to p130 and p107, although pRB/E2F4 complexes can also be found (Trimarchi and Lees, 2002). The repressive E2Fs do not have an N-terminal NLS, and shuttle between the nucleus and cytoplasm dependent on pocket protein binding. The most recently identified, and least well characterized E2Fs are E2F 6-8; these appear to function as repressors and are different from the previously described pRB family members in that they do not bind

pocket protein family members (de Bruin et al., 2003a; Di Stefano et al., 2003; Logan et al., 2004; Maiti et al., 2005; Morkel et al., 1997; Trimarchi et al., 1998).

Complexes between the pRB and E2F family members are dynamic, and change through the cell cycle. In quiescent cells, p130/E2F4 were found to be the predominant complex present (Ikeda et al., 1996; Richon et al., 1997). As cells proceed in the cell cycle, free E2F accumulates, and p130 levels decrease. This decrease in p130 protein levels is largely due to regulation of p130 protein stability caused by CDK4/6 mediated phosphorylation of p130 and subsequent recognition by the ubiquitin ligase SCF Skp2, targeting p130 for degradation (Tedesco et al., 2002). As cells enter S-phase, E2F is found complexed with p107 (Cao et al., 1992; Richon et al., 1997; Shirodkar et al., 1992). Promoter occupancy studies using chromatin immunoprecipitation coupled with microarray analyses have shown that E2F4 is replaced with E2F1-3 on many promoters with entry into S-phase (Takahashi et al., 2000).

E2F target genes encode critical cell cycle proteins, including cyclin A, cyclin E, cdk2, cdc2 and DNA replication factors (dihydrofolate reductase, thymidine kinase, DNA polymerase alpha, CDC6, Orc1) (Trimarchi and Lees, 2002). While E2F activity has been well-documented as being important for cell cycle, in a report in which chromatin immunprecipitation was coupled to DNA microarray analyses to identify promotor occupancy it was shown that E2F1 and E2F4 are found on promoters of genes in such diverse processes as replication and cell cycle control, as well as DNA damage, repair,

chromatin regulation and mitotic spindle checkpoint (Ren et al., 2002). Thus, E2Fs and their upstream regulators may have broader roles than initially thought.

pRB Binding Partners

pRB mediates repression by binding to regulators of chromatin. The best characterized of these interactions is the binding of pRB to histone deacetylases (HDACs) (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). Histones are basic proteins with N-terminal tails that can be modified by acetylation or methylation to alter chromatin structure (Turner, 2002). Acetylation can cause chromatin to adopt an open configuration more accessible to transcription factors. HDAC removes acetyl groups from histone tails, which regulates nucleosome function, making chromatin less accessible to transcription factor binding. Addition of the HDAC inhibitor trochostatin A could inhibit pRB-mediated repression of an E2F-regulated promoter (Brehm et al., 1998; Luo et al., 1998) suggesting that HDAC-mediated effects are important for pRB regulation of at least some targets.

Also, interactions between pRB and the hSWI/SNF nucleosome remodeling complexes may be important for repressive effects of pRB on chromatin (Harbour and Dean, 2000). SWI/SNF members are ATP-dependent mediators of nucleosome remodeling, that may act by repositioning histones (Narlikar et al., 2002). A model involving sequential displacement of different pRB partners depending on the extent of pRB phosphorylation has emerged (Harbour et al., 1999; Zhang et al., 2000). Complexes between pRB/E2F/HDAC/hSWI/SNF may repress promoters such as that of cyclin E. Phosphorylation of pRB by cyclinD/CDK4 leads to displacement of HDAC1 from the complex, allowing for expression of cyclin E, but not cyclin A. Under this model, pRB is

still bound and inhibiting the transactivation domain of E2F. Subsequent activation of CDK2/CyclinE activity leads to disruption of the pRB/E2F/hSWI/SNF complex and full activation of E2F targets, including cyclin A. This model helps explain the orchestration of stepwise roles for pRB in entry into S-phase, as well as later roles in cell cycle progression. Further verification, however, is needed to determine the accuracy of this model in vivo.

In addition to pRB-mediated regulation of histone acetlyation, immunoprecipitations of pRB have been found associated with histone methylase activity (Nielsen et al., 2001). pRB was shown to interact with a histone methylase SUV39H1 which methylates Lys9 of Histone H3 (Nielsen et al., 2001). Methylated Lys9 binds the heterochromatin regulator HP1 which is thought to regulate chromatin silencing. HP1 can also bind pRB, via a LxCxE motif (Nielsen et al., 2001). pRB- mediated recruitment of these repressor molecules could contribute to repression of pRB target genes, such as cyclin E. Methylation of Lys9 would require the absence of an acetyl group at this position. Thus, initial interactions of pRB with HDAC may mediate the stepwise deacetylation of Lys9 followed by the methylation of Lys9 to cause full repression.

Interestingly, pRB has been found in sites of heterochromatin that is induced in senescent cells. These senescence associated heterochromatin foci (SAHF) exhibited repression of pRB targets, and were associated with Histone H3 Lys9 methylation and HP1 binding (Narita et al., 2003). pRB may have roles in recruiting factors important in heterochromatin formation or maintenance. Interestingly, in rod photoreceptors, pRB has

been reported to be localized to a distinct form of heterochromatin, suggesting that the role of pRB in the regulation of chromatin structure may differ in a cell type and cell context dependent manner (Zhang et al., 2004a).

While much of the focus on pRB function has been tied to regulation of E2F activity, pRB can bind to many other proteins (Morris and Dyson, 2001). MDM2 has been reported to bind to the C-terminus of pRB, and ectopic MDM2 expression could regulate E2F activity (Dyer et al., 1981). While MDM2 has been well known to be the major regulator of the stability of the p53 tumor suppressor, it was only recently shown that MDM2 could also promote the ubiquitination and degradation of pRB (Uchida et al., 2005). pRB has been found to be acetylated via interactions with p300, also on the C-terminus. Acetylation was found to which favor pRB/MDM2 interaction, and decrease CDK-mediated pRB phosphorylation (Chan et al., 2001). pRB has also been proposed to bind to C-abl via the pRB C-terminus. This was reported to inhibit Abl kinase activity (Welch and Wang, 1993).

While E2F is the best understood pRB-regulated transcription factor, pRB also has been reported to regulate other transcription factors. pRB was demonstrated to bind to the osteogenic transcription factor CBFA1, which promotes osteogenic differentiation through direct effects on the promoters of osteogenic genes (Thomas et al., 2001). This is a clear example of pRB effects on transcriptional regulation in differentiation that is independent of E2F. Interactions between pRB and the microphthalmia-associated transcription factor (Mitf) in regulation of the p21 or tyrosinase promoter have also been

described (Carreira et al., 2005), as has interaction between pRB and CCAAT-enhancer binding proteins (C/EBPs) in regulation of fat differentiation (Chen et al., 1996) and interaction between pRB and MyoD in muscle differentiation (Gu et al., 1993). Interestingly the data with these tissue-specific differentiation factors implicate pRB as a positively activating transcriptional coactivator with tissue-specific roles in regulation of differentiation. Roles of pRB in differentiation control could contribute to the tumor suppressor function of pRB and a better understanding of this function of pRB in different tissue contexts is needed.

pRB and replication

In addition to roles in regulating the G1/S transition, pRB has also been linked more directly to DNA replication control. pRB has been localized to small discrete foci that encorporate BrdU and were suggested to be early sites of replication (Kennedy et al., 2000). Also, in response to DNA damage delivered during S-phase, pRB could be found to locate to known replication initiation sites (Avni et al., 2003). Interestingly, Rbf, a homologue of pRB in Drosophila was found to locate to origins of replications in complex with a drosophila origin recognition complex, and *Rbf* mutants exhibited failure in limiting replication (Bosco et al., 2001).

pRB and apoptosis inhibition

Outside of pRB function in the cell cycle, pRB has also been proposed to function as an inhibitor of apoptosis (Chau and Wang, 2003). pRB is a target of caspases activated during the apoptotic program (An and Dou, 1996). Intriguingly, analyses of mice with a

targeted mutation abolishing the main pRB caspases cleavage site revealed tissue-specific resistance to apoptosis, with clear resistance to TNF-alpha pathway mediated death, but not to DNA damage induced apoptosis (Chau et al., 2002). Experiments using this mutant allele suggested that pRB is a caspase-cleavage target important for amplification of the apoptotic signal before commitment to cellular death. The pRB pathway has been linked to the apoptotic program; many caspases (Nahle et al., 2002) as well as the critical apoptosis mediator Apaf1 (Moroni et al., 2001) are E2F transcriptional targets and their expression is activated in *Rb*-/- cells. Many other apoptotic targets has also been described as E2F target genes (Hershko et al., 2005; Hershko and Ginsberg, 2004). In cortical neuron cultures, DNA damage causes an induction in CDK activity, and pRB phosphorylation. Apoptosis is strikingly reduced using a chemical CDK inhibitor, or with expression of non-phosphorylatable pRB, implicating pRB in protection from apoptosis (Park et al., 2000).

Mouse phenotypes with Rb mutation

This section will describe some of the efforts used to determine the role of the pRB family *in vivo* in the mouse. Targeting of the *Rb* locus led to the surprising finding that *Rb*+/- mice never developed retinoblastomas, in contrast to germline *RB*+/- humans in which >90% develop retinoblastomas(Gallie et al., 1999). *Rb*+/- mice, however, were tumor prone, developing pituitary and thyroid tumors in which the remaining wild-type *Rb* allele was inactivated (Hu et al., 1994; Jacks et al., 1992). Homozygous *Rb* deletion in the mouse leads to lethality in midgestation revealing an essential role for this tumor suppressor gene in development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992).

The lethality was thought to be due to anemia stemming from erythropoietic defects. *Rb*-/- embryos also exhibited defects in proliferation and differentiation in the central and peripheral nervous systems, and the lens (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Lee et al., 1994; Morgenbesser et al., 1994). Unscheduled proliferation was coincident with high levels of apoptosis in each of these tissues. In the midgestation central nervous system, S-phase entry is normally confined to a location along the ventricles. However, in the pRB mutants, ectopic S-phase activity in the intermediate zone occurs in a widespread fashion. In addition, free E2F is elevated, and E2F target genes, such as *cyclin E* are overexpressed (Macleod et al., 1996). In the peripheral nervous system, Rb loss led to increased proliferation, apoptosis, as well as differentiation defects. Expression of differentiation markers such as betaII tubulin, TrkA and TrkB was greatly decreased in *Rb*-/- PNS (Lee et al., 1994). Similarly, in the lens, *Rb* deletion leads to unscheduled S-phase in lens fiber cells in the differentiating compartment. This was accompanied by apoptosis. Furthermore, expression of late stage lens differentiation markers such as beta and gamma-crystallin was markedly reduced (Morgenbesser et al., 1994). Thus, in many tissue compartments, pRB has a critical role in exit of the cell cycle and transition to a differentiated cell, with loss of pRB leading to unscheduled proliferation, failure in appropriate differentiation and apoptosis.

Compound mutant analyses were undertaken to dissect pathways leading to the phenotypes in the *Rb*-/- embryos. p53 is a critical mediator of apoptosis in response to diverse stimuli (Vousden, 2000). In *Rb*-/- embryos, p53 was overexpressed in the CNS and p53 binding activity was increased (Macleod et al., 1996). To determine if the

apoptosis in *Rb*-/- embryos is p53-dependent, compound *Rb/p53* null embryos were generated. Apoptosis in the *Rb*-deficient CNS and lens was completely rescued on a *p53*-/- background, while there was no rescue of apoptosis in the PNS and no rescue of the proliferative defects (Macleod et al., 1996; Morgenbesser et al., 1994). This led to a separation of the apoptosis and proliferation defects in the CNS. The *Rb*-mutant phenotypes have been further dissected to delineate the pathway to apoptosis in different tissues. For example, compound mutant analyses have shown that *Apaf1* is critical for apoptosis in the CNS and lens (Guo et al., 2001) while *Caspase3* is essential for apoptosis in the PNS (Simpson et al., 2001).

By crossing the *Rb* mutants into an *E2f1*-/- background it was shown that the lifespan of *Rb* mutant embryos could be extended, and both CNS apoptosis and inappropriate S-phase entry could be rescued (Tsai et al., 1998). Similar findings were made when *Rb* was crossed into an *E2f3*-/- background (Ziebold et al., 2001). These experiments illustrate the critical role of E2F members downstream of pRB in vivo. This led to the proposal that E2F activity due to inappropriate S-phase is upstream of both the proliferative defects and apoptosis in the *Rb* mutants. A search for a mediator between E2F activation and p53 was undertaken. A strong candidate was Arf, which is strongly induced at the RNA level by E2F activation (Bates et al., 1998; DeGregori et al., 1997) and is now a well established activator of p53 (Lowe and Sherr, 2003). However compound mutant *Rb/Arf* analyses did not reveal any role for *Arf* upstream of the CNS apoptosis in *Rb*-/- embryos (Tsai et al., 2002).

Rb-/- embryos also exhibit defects in erythropoiesis; the number of immature nucleated red blood cells was increased and mature enucleated erythrocytes was reduced in these embryos. Colony formation studies showed defects in erythrocyte progenitor differentiation, suggesting that there was an intrinsic defect in the progenitors (Jacks et al., 1992). However, there were also indications that non-cell-autonomous factors played a role. *Rb*-/- fetal livers could reconstitute an irradiated host, although some cells with defects in red blood cell enucleation were found (Hu et al., 1997). Also, in chimeric animals, *Rb*-deficient erythrocytes could differentiate normally, suggesting that wild-type cells can promote the normal development of *Rb*-/- erythrocytes (Maandag et al., 1994; Williams et al., 1994). Fetal liver macrophages are important in fetal liver erythropoiesis and were found reduced and defective in germline *Rb* mutants (Iavarone et al., 2004). Using a macrophage/erythrocyte co-culture system, in the presence of normal fetal liver macrophages, *Rb*-/- erythrocytes could enucleate normally (Iavarone et al., 2004). It is not clear, however, if there is an intrinsic defect in fetal liver macrophages with *Rb* mutation, or if the observed defects were due to defective macrophage development because of factors in the *Rb*-/- environment.

Since the studies of germline *Rb* mutants, it has become increasingly clear that many of the developmental phenotypes in these embryos were not cell-autonomous. Evidence that wild-type cells could affect the survival of *Rb*-/- cells in the CNS came from work in which *Rb*-/-:+/+ chimeric embryos were generated and survival of *Rb*-/- CNS cells in the presence of wild-type cells was found (Lipinski et al., 2001). The hypothesis was made that survival signals emanating from normal CNS cells contributed to the apoptosis.

However, the differential survival vs. death of *Rb*-/- neurons could have been due to more distantly originating signals. In chapter 2, I describe a hypothesis that such death signals were due to the anemic environment caused by the erythropoietic defect in *Rb*-/- embryos. Generation of animals with *Rb* deleted in the nervous system on a background of normal blood development led to the finding that CNS proliferation defects remained, but apoptosis levels were similar to wild-type at E13.5 (MacPherson et al., 2003). Similar survival of *Rb*-/- neurons was reported in a telencephalon-specific *Rb* knockout (Ferguson et al., 2002).

The discovery that *Rb*-/- embryos also have placental defects shed further light on the contribution of the environment to the phenotype of cells lacking *Rb*. It was found that extensive trophoblast stem cell proliferation contributed to defective placental transport function, which could contribute to a hypoxic environment (Wu et al., 2003). Furthermore, rescue of the placental defects through use of the Cre-loxP system to conditionally delete *Rb* in the embryo proper but not placenta led to survival until birth (de Bruin et al., 2003b). Other genes previously shown to genetically interact with *Rb* to lead to an extension of the survival of *Rb*-/- embryos (such as *E2f1* and *E2f3*) likely contribute to pRB function in the placenta. Another gene that genetically interacts with *Rb* in vivo is *ID2*. ID2 is an antagonist of helix-loop-helix transcription factors and a pRB-binding protein has been implicated in the pRB pathway. Overexpression of ID2 in cell culture could bypass a cell cycle block imposed by pRB (Iavarone et al., 1994). In vivo, compound *Rb/ID2* mutants survived until birth (Lasorella et al., 2000). It is not yet

clear if ID2 is broadly important downstream of pRB in vivo, or if ID2 has more tissue-specific roles, for example, in the placenta.

Functional overlap in the pocket protein family

While *Rb* mutation leads to lethality on a 126/C57Bl6 mixed background, inactivation of *p107* or *p130* on the same background does not lead to death or any overt phenotypes (Cobrinik et al., 1996; Lee et al., 1996). Loss of one pocket protein member may be compensated by the presence of the other members. Functional overlap between *Rb* and *p107* has been shown as compound *Rb/p107*-/- embryos died near E11.5, which is earlier than *Rb*-/- embryos. Also compound inactivation of both *p107* and *p130* led to perinatal lethality, with shortened limbs and bone defects (Cobrinik et al., 1996). Interestingly, genetic modifiers have striking effects on the phenotype of mice lacking p107 or p130. On a Balb/c background, *p107*-/- exhibit growth retardation and exhibit a myeloproliferative disorder (LeCouter et al., 1998a). Also, *p130*-/- animals on this background die during development in midgestation with neural tube defects (LeCouter et al., 1998b).

Functional overlap between *Rb* and *p107* extends to control of retinal development. Chimeric animals with both *Rb* and *p107* inactivated in ES cells were generated with low efficiency and 5 of 7 animals that survived into adulthood developed retinoblastoma (Robanus-Maandag et al., 1998). These tumors had histological similarities to inner-nuclear-layer retinal cells and showed evidence of differentiation along the amacrine cell lineage. The characteristics of these retinoblastomas are described further in the next

section. These results suggested that loss of pRB in the mouse does not lead to retinoblastoma because of compensation involving p107. Consistent with this idea, in many tissue culture cells lacking pRB, including MEFs, myocytes and keratinocytes, p107 was strongly upregulated (Hurford et al., 1997; Ruiz et al., 2004; Sage et al., 2003; Schneider et al., 1994). However, it remains to be determined if such an effect occurs in the absence of tissue culture stress; whole E13.5 *Rb*-/- embryos, or isolated brain lysates lacking pRB did not exhibit upreglation of p107 protein (Jiang et al., 1997). While illustrating the redundancy between pRB family members in tumor suppression, the *Rb/p107* chimera studies also indicated that retinoblastoma can indeed be studied in the mouse.

Part B: Retinoblastoma and retinal development

Retinoblastoma is one of the clearest examples of a tumor with developmental origins. In humans, retinoblastomas can happen during embryonic development (Maat-Kievit et al., 1993) and almost always occur in the first few years of life. The developing retina is the substrate for retinoblastoma-causing mutations and it is unknown why, during a certain time window, this tissue is exquisitely sensitive to tumorigenesis with *RB* mutation. This section will attempt to integrate some of our knowledge of retinoblastoma and the development of the retina,

Organization of the mature retina

The retina transduces light into neural signals, integrates visual input received across many cells and relays this information to the brain. The retina consists of three layers of cell bodies containing 7 major cell types separated by two plexiform layers rich in synaptic processes. The outer segments of photoreceptors (rods and cones) detect photons of light focused by the lens. Photoreceptors relay signals to bipolar neurons, the cell bodies of which locate to the inner nuclear layer, in a pathway that leads to retinal ganglion cells, located in the innermost, ganglion cell layer. Horizontal and amacrine interneurons modulate these signals. The axons of the retinal ganglion cells travel along the optic nerve, transmitting information to the brain. There is a glial cell type in the retina that spans all retinal layers, the Muller glia. The Muller glia become activated in response to injury and may play a role in supporting other cells in response to damage, although the function of the Muller glia is not clear. In addition to these 7 cell types, (which all have a common developmental origin), there are astrocytes in the inner retina that have a different origin, migrating from the optic nerve (Watanabe and Raff, 1988). The circuitry of the retina is complex and within most of the described retinal cell classes, there are numerous distinct subtypes that can be differentiated based on both morphological criteria, as well as expression of specific markers (Masland, 2001).

Retinoblastoma cell type characteristics

There have been numerous studies of the cell-type composition of human retinoblastomas. Many retinoblastomas exhibit differentiated areas with the presence of "Flexner-Wintersteiner" rosettes, and "fleurettes" which have both histological and

ultrastructural similarities to photoreceptors (Ts'o et al., 1970a; Ts'o et al., 1970b). Also, another type of rosette, Homer-Wright rosettes, indicative of neuroblastic differentiation is also often found in retinoblastomas. Characteristics of retinoblastoma cell lines have been used to gain insight into the retinoblastoma cell of origin. It has been proposed that retinoblastoma derives from a primitive neuroectodermal cell with the potential to differentiate into photoreceptor or glial cells (Kyritsis et al, 1984). This was due to the in vitro characteristics in the Y79 retinoblastoma cell line, which could be chemically induced to differentiate into cells with glial characteristics, expressing GFAP, or neuronal characteristics, expressing Neuronal Specific Enolase (Kyritsis et al., 1984). Study of cell lines derived from retinoblastomas demonstrated that retinoblastomas expressed mRNA for cone, but not rod cGMP phosphodiesterase, as well as cone transducins (Bogenmann et al., 1988; Hurwitz et al., 1990). This suggested that retinoblastomas derived specifically from a cone lineage cell. However, there has been debate as to which types of photoreceptors are found in retinoblastomas. Di Polo and Farber, (1995) found evidence of both rod and cone phosphodiesterase transcripts in the Y79 retinoblastoma cell line. While the properties of cell lines derived from human retinoblastomas may provide some insight into the tumor originating cell, it is important to note that retinoblastoma cell lines have been difficult to generate, and genetic changes may occur in the establishment of the line and the passaging of the cell lines. Also, different cell types may preferentially be able to survive the culturing conditions even in early passage retinoblastoma lines. Thus, elucidation of the retinoblastoma cell-type characteristics in retinoblastomas that have been not cultured is important.

Many groups have performed immunohistochemical marker analysis on paraffin embedded retinoblastoma samples to examine the properties of tumor cells. The rod photoreceptor marker rhodopsin was found in Flexner-Wintersteiner rosettes in most tumors examined (Vrabec et al 1989, Donoso et al, 1986). Also interphotoreceptor retinoid binding protein (IRBP), present in rods and cones (Bunt-Milam and Saari, 1983) has been reported in retinoblastomas in many studies (Bernstein et al., 1994; Fong et al., 1988; Rodrigues et al., 1992). In a series of paraffin embedded retinoblastomas, both rod and cone markers were expressed in many retinoblastomas (Gonzalez-Fernandez et al., 1992). The question of whether glial cell characteristics are present in retinoblastoma tumor cells has not been resolved. Positive immunostaining for glial markers such as glia fibrillary acidic protein (GFAP) has been interpreted as reactive gliosis from non-tumor cells ((Perentes et al., 1987) or as indicative of neoplastic Muller cells (Nork et al., 1995) although no clear proof of these being neoplastic cells has been shown. Overall, retinoblastomas appear largely composed of cells with undifferentiated histology as well as differentiated areas in which rod and cone markers can sometimes be found to be expressed. The data suggest an origin in a multipotential cell but the extent to which these cells are multipotent requires further examination.

Retinoblastomas from *Rb/p107* chimeric mice developed between PND15 to 4 months of age and exhibited characteristics of amacrine cells, staining positively for the amacrine marker syntaxin. Some cells stained positively with GFAP, possibly due to reactive gliosis, but no staining for neurofilament (NF), which recognizes ganglion cells and horizontal cells was found. These tumors exhibited neuronal character, staining for

Neuronal Specific Enolase. An important question is whether the murine retinoblastomasa are similar or different entities compared to human retinoblastomas. Both human (Gallie et al., 1999) and the murine retinoblastomas (Robanus-Maandag et al., 1998) appear to emerge from the inner nuclear layer. They also have histological similarities, including the presence of Homer-Wright rosettes, and neuronal character. The absence of Flexner-Wintersteiner rosettes in murine tumors and cell-type characteristics attributed to human vs. mouse tumors were different. However, there have been hints of amacrine differentiation in human retinoblastoma; Tsokos et al (Tsokos et al., 1986) report the production of catecholamines, which are produced by amacrine cells, in a retinoblastoma cell line. Also, only one photoreceptor marker was examined in the murine *Rb/p107* chimera study. Thus, more work is needed to more conclusively delineate the similarities and differences between murine and human retinoblastomas.

Development of the retina

The retina develops from anterior tube neurepithelium that undergoes a lateral bulge following the closure of the neural tube (reviewed in (Chow and Lang, 2001). This bulge forms the optic vesicles that evaginate to approach the surface ectoderm. Signals between the surface ectoderm and the optic vesicle are involved in subsequent invagination of the central part of the optic vessel, forming the optic cup. The optic cup is bilayered, with an inner layer (neural retina) and outer layer (retinal pigment epithelium). Retinal progenitors divide along the outer edge of the neural retina in an area termed the retinal ventricular zone (Cepko et al., 1996). Different phases of the cell cycle occur in different spatial locations in the retina. While mitoses occur along the

retinal ventricular zone, an interkinetic nuclear migration occurs, such that nuclei migrate away from this zone to undergo G1, S and G2 cell cycle stages and return for the subsequent mitosis (Young, 1985b). Cells exit the cell cycle, migrate to their ultimate destination and establish appropriate synaptic connections. Either both daughters can exit the cell cycle, or an asymmetric division can occur in which one cell may exit, while the other may stay in a progenitor-like state.

Early studies using H3-thymidine incorporation in rodents have demonstrated that there is a characteristic order in which retinal cell types are generated (Young, 1985a). In an overlapping fashion, retinal ganglion cells, horizontal cells, cones and amacrine cells are generated first, largely during embryogenesis, while bipolar cells, rods and Muller glia are generated in later stages of retinal development. In the mouse, proliferation is completed by postnatal day 11 (PND11) (Young, 1985b). There is a central to peripheral wave of differentiation in the mammalian retina; S-phase entry in central retina is complete at PND6 while the last cycling cells, restricted to the periphery of the retina, are found at PND11 (Young, 1985b).

To determine if mature retinal cell types were derived from progenitor cells that were multipotent, or unipotent, lineage-tracing studies were undertaken. Replication incompetent retroviral vectors were used to mark clones in the postnatal retina, revealing that a single cell can produce a clone containing very different cell types (Turner and Cepko 1987). Indeed, 2-cell clone analyses revealed that even a cell undergoing it's final mitosis can produce a Muller glial cell and a rod photoreceptor (Turner and Cepko,

1987). Infection of cells in the embryonic retina (Turner et al., 1990) revealed that nearly all cell types can be formed from a single multipotent progenitor. Importantly, at E13 and E14, over 99% of the clones containing more than one cell type were multipotential, and at P0, over 65% of the clones were multipotential. These studies have indicated that mature retinal cell types derive from progenitors that retain multipotency to a large extent even very late in retinal development. There is a difference in potency in early vs. late retinas, with some restriction on the cell types that form later in development.

Environment vs. intrinsic differences in cell fate

There is good evidence for important roles for both environmental factors, as well as intrinsic changes in competency regulating the retinal cell type produced. Support for the concept that the environment is important in regulating cell fate comes from co-culture studies. When embryonic rat retinal cells were cultured with postnatal cells, the embryonic cells produced substantially more rod photoreceptors than when cultured alone (Watanabe and Raff, 1990). Also, young retinas at E16.5 had a decreased ability to form amacrine cells when co-cultured with older cells (Belliveau and Cepko, 1999). This was found to be due to inhibition from more mature amacrine cells, implicating a feedback loop regulating amacrine cell genesis. It has also been found that addition of Ciliary Neurotrophic Factor (CNTF) to rat retinal explants led to an increase in bipolar cells and decrease in rods (Ezzeddine et al., 1997). This corresponded to a cell fate change from rods to bipolar cells in the presence of a diffusible factor. In addition to supporting a role for the environment in cell fate, these experiments also suggest that there is plasticity in commitment to cell-type choice, even in post-mitotic cells.

Intrinsic changes in the cell over time also contribute to cell fate decisions. For example, mitotic cells expressing the marker VC1.1 preferentially develop into amacrine cells or horizontal cells, but not other retinal cell types (Alexiades and Cepko, 1997). These data also indicate that there is heterogeneity in the progenitor cell population, and the degree to which progenitors are heterogenous, with different competencies is not well understood. Heterogeneity in progenitor cells extends to differences in regulation of the pRB pathway; different subsets of progenitor cells express the CDKIs p27kip1 vs. p57kip2 and this has been implicated in regulating cell cycle exit for specific cells (Dyer and Cepko, 2001a).

Cell Death and Retinal Development

Analyses of cell death in the mouse retina has revealed a reproducible pattern of death which differs with retinal cell type (Young, 1984). The most numerous cell in the retina is the rod photoreceptor (Jeon et al., 1998). For rods, a particularily sensitive period occurs near PND7/ 8 when the development of the outer plexiform layer traps some rods on the inner side, and these must migrate to the outer section of the inner plexiform layer. This wave of migration is associated with significant rod cell death. Nevertheless, only a very small proportion of rods (~5%) that are born undergo apoptosis (Young, 1984). Other cell types also have characteristic waves of cell death. It has been estimated that about 50% of ganglion, bipolar and amacrine cells undergo apoptosis (Young, 1984). Neuronal survival may depend on neurons obtaining adequate trophic support and forming appropriate synaptic connections (Cellerino et al., 2000). Overexpression of the

anti-apoptotic Bcl2 under control of the neuronal specific enolase promoter substantially reduced ganglion cell death during development (Strettoi and Volpini, 2002). The retina also undergoes apoptosis in response to stress. In response to gamma-irradiation, the developing murine retina has been shown to undergo p53-dependent apoptosis (Borges et al, 2004). While the response to cellular stress in some situations requires p53, in other situations, apoptosis is p53-independent. Light-induced photoreceptor apoptosis was found to be p53-independent, for example (Marti et al., 1998).

Cell Death and retinoblastoma

The idea that suppression of cell death in the retina could contribute to tumor development has been supported by studies of DNA tumor viral protein expression in the retina. In an attempt to model retinoblastoma, Howes et al (1994) (Howes et al., 1994) expressed HPV E7 under the photoreceptor-specific IRBP promoter. The E7 oncoprotein binds and functionally inactivates the pRB family, and, under IRBP promoter control, photoreceptor apoptosis resulted. However, on a *p53*-/- background, photoreceptor tumors emerged and this was attributed to a rescue in apoptosis with p53 loss. This suggested that p53-dependent apoptosis may have to be suppressed for retinoblastomas to develop. This is also consistent with reports that SV40 early region expression under the IRBP promoter leads to photoreceptor layer tumors (Marcus et al., 1996) as the expression of SV40 Large T antigen should inactivate both p53 and the pRB family. Expression of SV40 early region in a β leutenizing-hormine (β-LH) transgenic (in which retinal expression was a consequence of transgene integration site and not normal β-LH expression) led to the formation of tumors with neuronal properties emerging from

the inner layer (Windle et al., 1990). Targeting DNA tumor viral proteins to different retinal cell compartments leads to different tumors. A major question is whether these models recapitulate tumorigenesis originating from the cell type relevant to human retinoblastoma. Chimeric animals with cells lacking *Rb* exhibited photoreceptor apoptosis (Maandag et al., 1994). Similarly, murine retinoblastomas in *Rb/p107* chimeric animals arose out of highly apoptotic embryonic retinas (Robanus-Maandag et al., 1998). It has been hypothesized that suppression of apoptosis may cooperate with *RB* loss to lead to retinoblastoma (Gallie et al., 1999). However, human retinoblastoma tumors are extremely apoptotic tumors and *p53* mutations have not been found in primary retinoblastomas (Gallie et al., 1999). A critical question is whether the apoptosis in the *Rb*-/-:+/+ chimeras is p53-dependent, and whether apoptosis must be suppressed for retinoblastomas to develop.

Rb pathway and retinal development

Ultimately, the control of progenitor cell proliferation and exit from the cell cycle feeds through regulation of the pRB family. Early in retinal development, with expansion of retinal progenitors, upstream regulators of the pocket protein family are thought to maintain the pocket proteins in an inactive state through regulation of phosphorylation. Perhaps most important in the developing retina is cyclin D1, which is the major D-type cyclin in the developing retina. Cyclin D1 is expressed at high levels in the developing retina in the vast majority of proliferating cells (Sicinski et al., 1995). Deletion of *cyclin D1* leads to striking retinal hypoplasia, indicating a critical role of this upstream pRB regulator in retinal development (Fantl et al., 1995; Sicinski et al., 1995). Both decreased

mitoses as well as increased cell death contributed to the hypocellular *cyclin D1*-/-retinas (Ma et al., 1998). The retinal effects of cyclin D1 loss are likely due to regulation of the Rb pathway; *Cyclin D1*-/- retinas exhibited hypophosphorylation of pRB, and the *cyclin D1*-/- retinal phenotypes could largely be rescued by co-inactivation of the CDKI *p27*, which also partially rescued pRB phosphorylation (Tong and Pollard, 2001). Another contributing factor allowing for high levels of proliferation early in retinal development involves the repression of CDKI expression. Expression of various CDKI's, including p27 is suppressed early in retinal development by the homeodomain factor Six6 acting in concert with Dach corepressors which may allow for expansion of progenitor cells (Li et al., 2002). As retinal development proceeds, an increasing proportion of cells become postmitotic. Different cell types may use different means to exit the cell cycle; some cells increase the expression of CDKIs. Interestingly, this occurs in a different fashion in different progenitors, for example a subset of cells upregulate p27 or p57 to exit the cell cycle (Dyer and Cepko, 2001a; Dyer and Cepko, 2001b; Levine et al., 2000). In *p27*-/- mice, the period of retinogenesis was extended, consistent with a role in cell cycle exit (Levine et al., 2000). Also, ectopic expression of p27 could enforce premature cell cycle exit (Dyer and Cepko, 2001a). Expression of INK4d also plays a role in retinal progenitor cell cycle exit, with *INK4d*-/- animals also exhibiting an extension of the period of retinogenesis which is accompanied by apoptosis (Cunningham et al., 2002). In some retinal neurons, both p27 and Ink4d are coexpressed, suggesting that they both may cooperate to induce cell cycle exit, or help to block cell cycle reentry (Cunningham et al., 2002). In addition to CDKI upregulation, levels of cyclin D1 decrease as cells exit the cell cycle (Dyer and Cepko, 2001a). Thus, upregulation of CDKIs coupled with

downregulation of cyclin D1 appear to cooperate in conferring cell cycle exit, presumably via their regulation of pRB family activity.

pRB function in the mouse retina

In humans, pRB has been found to be highly expressed in some photoreceptors, in bipolar cells and cells in the ganglion cell layer (Yuge et al., 1995). Transient expression of pRB during human retinal development has not been examined, and it is possible that pRB could still play a physiologic role in cells in which expression is below the level of detection by immunohistochemistry. In murine embryogenesis, Rb expression between E13.5-E17.5 has been described by in situ hybridization, with high expression specifically in the retinal ganglion cell layer (Jiang et al., 1997). In newborn amimals (PND0) pRB was found expressed in the ganglion cell layer, as well as in the neuroblastic layer, rich in progenitor cells (Zhang et al., 2004a). By labeling cells with 3H thymidine before assaying for pRB expression, it was found that 85% of cells in S-phase were positve for pRB. This is consistent with recent finding of increased Rb expression in progenitor enriched retinal cells (Livesey et al., 2004). At later stages, pRB was found in postmitotic neurons in the inner nuclear layer as well as in photoreceptors (Zhang et al., 2004a). A full characterization of pRB, p107 and p130 expression through development in specific cell types remains to be reported. Until recently, there has been little functional insight into the role of pRB in the murine retina, given the early lethality of germline *Rb* mutants and lack of tumor phenotype in *Rb*+/- animals. Also, the difficulties in generating adult chimeric animals lacking *Rb* and *p107* hindered analyses of the genesis of murine retinoblastoma. However, recent advances using tissue specific

gene inactivation in the retina have allowed for a better understanding of pRB function in the retina, and better models of retinoblastoma (Chen et al., 2004; MacPherson et al., 2004; Zhang et al., 2004a; Zhang et al., 2004b). Chapter 3 describes my work in this area; integration of this work with the recent independent work of the Dyer and Bremner labs on pRB function in the retina will be discussed Chapters 5.

Part C: The p53 tumor suppressor

The origins to the discovery of the *p53* gene lie in studies of viral oncoproteins: p53 was identified as a cellular protein found in complex with large T antigen in cells transformed with SV40 T-Ag (Lane and Crawford, 1979; Linzer and Levine, 1979). Following cloning of the p53 cDNA (Bienz et al., 1984; Oren and Levine, 1983), initial experiments indicated that p53 had oncogenic properties. p53 could cooperate with mutant oncogenic Ha-Ras in transformation of primary rat embryo fibroblasts (Eliyahu et al., 1984; Parada et al., 1984). However, it was later realized that point mutations occurred in some of the original p53 cDNAs that were cloned and that mutated p53 functioned in a dominant manner. We now know that p53 functions as a tetramer, such that encorporation of wild-type p53 into tetramers with also containing mutant p53 can lead to dominant-negative effects (Milner and Medcalf, 1991; Milner et al., 1991). Finlay et al. (1989) showed that wild-type p53 actually functions to suppress transformation induced by oncogenes. Aided by discovery of *p53* point mutations in human tumors, (Baker et al., 1989) p53 function as a tumor suppressor was demonstrated. Germline mutations in *p53* were subsequently found in the cancer predisposing Li-Fraumeni Syndrome (Malkin et al., 1990; Srivastava et al., 1990). Li-Fraumeni patients were prone to tumors such as sarcomas, breast

carcinoma, leukemia, osteosarcoma, brain, melanoma, adrenocortical carcinoma and other cancers. Analyses of spontaneous tumors has since revealed that *p53* is the most frequently mutated tumor suppressor known; *p53* mutations are found in over half of human tumors. (Bartek et al., 1991; Greenblatt et al., 1994; Hollstein et al., 1991; Vogelstein et al., 2000). Thus, p53 has been firmly established as a critical, and broadly acting suppressor of human cancer.

The p53 protein

p53 acts as a node through which cellular stress signals are integrated into decisions to undergo diverse responses such as cell cycle arrest, apoptosis, cellular senescence and activation of DNA repair (reviewed in (Vogelstein et al., 2000)). *p53* encodes a transcription factor that largely functions by activating the expression of a diverse group of downstream target genes. Human p53 protein has 393 amino acids with four major functional domains; an N-terminal transactivation domain, a central sequence-specific DNA binding domain, a C-terminal oligomerization domain that mediates p53 tetramerization, and an extreme C-terminal regulatory domain (Levine, 1997). The DNA binding domain is the most frequent site of mutations. The two most frequently mutated residues on p53, Arg248 and Arg273 directly contact DNA, and other frequently mutated residues stabilize the structure of the p53 DNA binding surface (Cho et al., 1994). The location of mutation hotspots for p53 in tumors support the idea that DNA binding is critical for p53 tumor suppressor function.

The peripheral regions of p53, while less frequently mutated in cancer, are also critical for p53 function. The N-terminal transactivation domain binds to components of the basal transcriptional machinery including subunits of TFIID (Lu and Levine, 1995; Lutzker and Levine, 1996), the TATA-box binding ptotein (TBP) (Horikoshi et al., 1995) as well as the transcriptional coactivators p300/CBP (Avantaggiati et al., 1997; Gu et al., 1997; Lill et al., 1997). Both recruitment of basal transcription machinery, as well as modulation of chromatin structure through coactivator activity have been implicated in conferring p53-mediated effects on transcription of target genes. The N-terminus is also the region of p53 that binds to the major negative regulator of p53, MDM2.

The C-terminus contains a nuclear localization signal (NLS) (Shaulsky et al., 1990) as well as a nuclear export signal (NES) (Stommel et al., 1999). Interestingly, the NES lies with the tetramerization domain, such that in tetramerization may mask this signal to promote accumulation of tetramers in the nucleus. The C-terminus also contains sequences proposed to negatively regulate p53 DNA binding activity. Deletion of this putative negative regulatory region, or addition of an antibody to the C-terminus could strongly increase sequence-specific DNA binding activity (Hupp et al., 1992). Original models proposed that p53 may require C-terminal modifications to convert p53 from a latent to an active form. p53 was found to be acetylated at the C-terminus on residues including Lys320 and Lys382 in response to DNA damage, and this was associated with increased sequence specific binding and transactivation ability (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). Both the histone acetyltransferases p300, as well as P/CAF were shown to acetylate p53. However these studies examined the effects of

p53 acetylation on p53 binding to small oligonucleotides, in gel mobility shift assays, and there is evidence that p53 acetylation does not increase p53 DNA binding on full length promoters (Espinosa and Emerson, 2001). Using an in vitro transcription assay, p300 addition did not increase p53 DNA binding, but increased p53 transactivation of the p21 promoter via acetylation of nucleosomes and consequent altered chromatin structure (Espinosa and Emerson, 2001). There is significant debate over the role of C-terminal acetylation of p53, and whether the C-terminus has an inhibitory role in vivo (Prives and Manley, 2001).

Negative Regulation of p53 via MDM2

MDM2 is a critical negative regulator of p53 that regulates p53 transactivation, protein stability and subcellular localization. The N-terminus of MDM2 blocks the transactivation domain of p53, impairing p53 function as a transcription factor (Momand et al., 1992). The crystal structure of the MDM2 N-terminus bound to a peptide from the region of p53 that binds MDM2 revealed that three hydrophobic residues known to be critical for p53 transactivation bind deep in an MDM2 cleft as an alpha helix (Kussie et al., 1996). Mdm2 functions as a RING-finger containing E3 ubiquitin ligase, which mediates the ubiquitination and degradation of p53 via the 26S proteasome (Hupp et al., 1992). In the ubiquitination pathway, an E1 enzyme binds and activates the 76 amino acid ubiquitin, (Lee and Peter, 2003). An E2 ubuitination-conjugating enzyme transfers the activated ubiquitin to the E3 ubiquitin ligase, which confers specificity in transferring ubiquitin to its substrate. Polyubiquitination, involving transfer of a chain of multiple ubiquitin molecules is needed to confer degradation while monoubiquitination often has

other biological effects. *In vitro* ubiquitination assays demonstrated that lower levels of MDM2 conferred monoubiquitination on multiple p53 lysine residues, while higher levels were needed for polyubiquitination (Li et al., 2003). It is also possible that MDM2 cooperates with other proteins in mediating polyubiquitination; for example p300 was reported to cooperate with MDM2 in p53 polyubiquitination (Grossman et al., 2003). MDM2 is also involved in the export of p53 from the nucleus to the cytoplasm (Roth et al., 1998; Tao and Levine, 1999). Interestingly, monoubiquitination promoted p53 export to the cytoplasm, suggesting that MDM2 ubiquitin ligase activity may control both p53 subcellular localization and degradation (Li et al., 2003). MDM2 is also a p53 target gene that acts in a negative regulatory loop to control p53 levels (Barak et al., 1993). The importance of MDM2 in regulating p53 is illustrated in the phenotype of the MDM2 knockout mouse. *MDM2*-/- animals die very early in embryogenesis. This phenotype is dramatically rescued on a *p53*-/- background as compound mutants survive into adulthood (Jones et al., 1995; Montes de Oca Luna et al., 1995). This suggests that lethality in *MDM2*-/- animals is due to activated p53 and support the idea that MDM2 is a critical negative regulator of p53 in vivo. An MDM2 relative, MDMX is less well-characterized but also appears to negatively regulate p53 function (Shvarts et al., 1996). In contrast to MDM2, MDMX lacks ubiquitin ligase activity, and has not been implicated in regulation of p53 localization or degradation. MDMX does, however, bind and inhibit the p53 transactivation domain. The importance of MDMX in p53 regulation was shown in vivo as the early lethality of *MDMX*-/- embryos was also rescued on a p53-/- background (Parant et al., 2001). While the role of *MDMX* in human cancers is still being determined, *MDM2* has clearly been found amplified in tumors in which p53

function is maintained. For example, in sarcomas, *MDM2* was amplified in over a third of tumors that retained intact *p53* (Oliner et al., 1992).

Upstream activation of p53

p53-activating stresses involve DNA breaks, UV irradiation, hyperproliferative signals, hypoxia, nucleoside triphosphate depletion, etc (Reviewed in (Vogelstein et al., 2000)). These stresses act to hinder the MDM2/p53 interactions allowing p53 stabilization and also act to increase p53 transactivation ability. The method in which this p53/MDM2 interaction is disrupted differs depending on the activating stress.

Oncogenic signals mediated by Ras, myc, adenovirus E1A activate p53 in large part by inducing the expression of ARF (de Stanchina et al., 1998; Palmero et al., 1998; Zindy et al., 1998). ARF binds MDM2, and is thought to function by both inhibiting the ubiquitin ligase activity of MDM2 (Honda and Yasuda, 1999) and by localizing MDM2 in the nucleolus (Weber et al., 1999). There is, however, debate as to which this nucleolar localization of MDM2 is needed for the inhibitory function of Arf (Korgaonkar et al., 2002; Llanos et al., 2001). As mentioned earlier, the *ARF* locus, which also includes the *p16INK4a* gene, is frequently disrupted in human cancers. Thus, the p53 pathway can be disrupted via *p53* mutation or *ARF* inactivation, or amplification of *MDM2*. Other events downstream of oncogenes are also important for p53 activation. For example, Ras can induce the expression of promyelocytic leukemia (PML) protein (Ferbeyre et al., 2000). This contributes to the formation of a PML/p300/p53 complex in nuclear bodies that appears important for increasing p53 transactivation ability (Pearson et al., 2000).

The best-characterized pathway leading to p53 activation is in response to DNA damage. Following DNA damage p53 undergoes numerous posttranslational modifications including phosphorylation and acetyation events that contribute to p53 protein stability and to p53 transactivation function. In response to ionizing radiation (IR), which induces double stranded DNA breaks, the activity of the ATM (ataxia-telangectasia-mutated) kinase increases (Canman et al., 1998). ATM phosphorylates various proteins involved in both double strand break repair and checkpoint function. For example, ATM phosphorylates the histone protein H2AX (Burma et al., 2001; Stiff et al., 2004) which is important for the recruitment of repair components to sites of damage (reviewed in (Motoyama and Naka, 2004). ATM also phosphorylates MDM2 which may hinder p53 nuclear export (Maya et al., 2001). ATM promotes the activity of other p53 regulators and is also thought to directly phosphorylate p53.

p53 phosphorylation

In response to ionizing radiation, phosphopeptide mapping studies examining endogenous p53 revealed that Ser 15 is phosphorylated following DNA damage (Siliciano et al., 1997). Furthermore, Ser15 phosphorylation was significantly delayed in lymphoblasts from patients with ataxia-telangestasia that contained mutations in *ATM* (Siliciano et al., 1997). ATM is thought to be critical for the early wave of Ser15 phosphorylation found following IR treatment (Banin et al., 1998; Canman et al., 1998). Consistent with the thought that ATM directly phosphorylates Ser15, the LSQE site around Ser15 is similar to an optimal ATM phosphorylation site identified through an

oriented peptide library search (O'Neill et al., 2000). In response to UV irradiation, which causes the formation of bulky DNA lesions, the ATR (ataxia-telangectasia related) kinase may function in a manner analogous to ATM to phosphorylate p53 on Ser15 (Tibbetts et al., 1999). Phosphorylation on Ser15 was initially proposed to confer p53 stabilization by displacing MDM2 (Shieh et al., 1997). However, subsequent studies have reported that Ser15 phosphorylation does not disrupt p53/MDM2 interaction but contributes to p53 transactivation function (Dumaz and Meek, 1999). This may occur via recruitment of the acetyltransferase CPB/p300 (Lambert et al., 1998). Consistent with a role for p53 Ser15 phosphorylation in conferring transactivaiton function, mice engineered to have a mutation converting the murine equivalent of this residue Ser18 to Ala exhibited normal p53 stabilization (Chao et al., 2003). Also, cells from these mice did exhibit defective transactivation function and defective C-terminal acetylation.

ATM also phosphorylates and activates the upstream p53-regulator Chk2 kinase on Thr68 (Matsuoka et al., 2000). This phosphorylation promotes the oligomerization and transphosphorylation of Chk2 (Ahn et al., 2002; Xu et al., 2002). Chk2 is genetically upstream of p53, as *Chk2*-/- cells have impaired p53 responses (Hirao et al., 2002; Hirao et al., 2000; Jack et al., 2002; Takai et al., 2002). Phospho-specific antibodies have been used to demonstrate that Ser20 of p53 is phosphorylated following DNA damage (Craig et al., 1999; Shieh et al., 1999). Chk2 can phosphorylate p53 in vitro on Ser20, and other residues, and the impairment of p53 stabilization with Chk2 deficiency was proposed to be due to effects on Ser20 (Chehab et al., 2000; Shieh et al., 2000). Ser20 lies in the region of p53 that interacts with MDM2, and phosphorylation at this site has been

predicted to disrupt p53/MDM2 interaction (Chehab et al., 1999; Kussie et al., 1996). Biochemical data support this as Ser20Ala mutants were more susceptible to destabilization via MDM2, and Ser20Ala mutants exhibited increased interactions with MDM2 (Unger et al., 1999a). Also, p53-dependent apoptosis was compromised with S20A mutation (Unger et al., 1999b). It is not clear if Chk2 phosphorylates p53 on Ser 20 or other residues in vivo. Studies using peptide libraries to search for optimal Chk2 phosphorylation motifs did not reveal sites similar to the sequences surrounding Ser20 of p53 (O'Neill et al., 2002). However, it has been demonstrated that the tetramerization domain of p53 is required for Chk2 –mediated phosphorylation of p53 in vitro (Shieh et al., 1999). Thus, the critical elements in the structure of tetrameric p53 needed for Chk2 phosphorylation may not be reflected in the phosphorylation of small peptides. Consistent with this idea, docking sites on the p53 DNA binding domain contributed to Chk2 phosphorylation of p53 on Ser20 in vitro (Craig et al., 2003). Inactivation of Chk2 expression using engineered zinc finger transcription factors (engineered to specifically repress the Chk2 promoter) led to decreased p53 Ser20 phosphorylation (Tan et al., 2003). Also, cells from *Chk2*-/- mice exhibited impairment in p53 stabilization (Hirao et al., 2000; Takai et al., 2002). However, countering these findings, targeted inactivation of *CHK2* in a human colon cancer line did not impair p53 phosphorylation on Ser 20 or p53 stabilization, clearly indicating that Chk2 is not required for Ser20 phosphorylation in these cells (Jallepalli et al., 2003). Besides Chk2, other kinases have been implicated in conferring Ser20 phosphorylation including JNK, MAPKAP2, Plk3 and Chk1 (She et al., 2002; Shieh et al., 1999; Xie et al., 2001). In summary, while there is good evidence that Ser20 phosphorylation is important for p53 protein stabilization under some conditions,

different experimental conditions lead to differing effects of this mutation in vitro, and the identity of the in vivo Ser20 kinase is not clear.

In addition to Ser15 and Ser 20, many other sites of phosphorylation on p53 have been reported, and some of these may also be important for p53 stabilization. JNK has also been implicated in conferring p53 stability via phosphorylation of Thr81 (Buschmann et al., 2001; Fuchs et al., 1998). On the N-terminus, other p53 residues including Ser 6, 9, 33, 37, 46 and Thr 18, 55 have been found phosphorylated through the use of phospho-specific antibodies (Dumaz and Meek, 1999; Giaccia and Kastan, 1998; Khanna et al., 1998; Meek, 1998). On the human C-terminus, Ser315, 378, 389 and 392 have been reported phosphorylated (Hupp et al., 1992; Siliciano et al., 1997). Kinases implicated in vitro in p53 phosphorylation include CK1 (Ser6, 9, Thr18), p38 (Ser33, 46), PKC (Ser 376, 378), DNA-PK (Ser15, Ser37) CK2 (Ser392), HIPk2 (Ser46), Erk2 (Thr55) (Reviewed in (Bode and Dong, 2004; Brooks and Gu, 2003). In very few cases has the in vivo relevance of these events been determined.

Other p53 modifications

p53 is modified in myriad ways at the posttranscriptional level. In addition to ubuiquitination, phosphorylation and acetylation, p53 has been reported to undergo many other modifications including neddylation, sumoylation, methylation and glycosylation (reviewed in (Bode and Dong, 2004)). p53 neddylation and sumoylation each involve the transfer of a ubiquitin-like molecule to p53. Neddylation of p53 involves the transfer of NEDD8 to C-terminal lysines (Lys 370, 372, 373) of p53. p53 neddylation was promoted

by MDM2 and caused a decrease in p53 transactivation ability (Xirodimas et al., 2004). p53 has also been found to be modified by conjugation to SUMO-1 at Lys386, which increased p53 transactivation ability (Gostissa et al., 1999; Rodriguez et al., 1999). Methylation of p53 on a single C-terminal lysine 372 by the set9 methylatransferase occurs following DNA damage and contributes to p53 stabilization (Chuikov et al, 2004). p53 function is tightly regulated, largely via signaling pathways that converge on p53 modifications. The ultimate effect of convergence of these pathways following stress is the formation of an active, stable transcription factor that can initiate responses such as cell cycle arrest or apoptosis.

Effects of p53 activation

Understanding the control of the decision to undergo responses such as cell cycle arrest vs. apoptosis is a central issue in the p53 field. A number of factors contribute to this decision. Cell types differ in their sensitivity to apoptosis vs. cell cycle arrest. For example, thymocytes have been demonstrated to undergo p53-dependent apoptosis in response to IR (Lowe et al., 1993) while mouse embryonic fibroblasts (MEFs) respond by undergoing arrest. Proliferating cells in the intestinal crypts, or in the developing central nervous system are also sensitive to p53-dependent apoptosis (Clarke et al., 1994; Herzog et al., 1998). Interestingly, primary MEFs respond to IR by undergoing arrest; however, if E1A is used to inactivate the RB family, disrupting proliferation control, these cells become sensitive to p53-dependent apoptosis (Lowe et al., 1994b). The factors in different cell types that confer sensitivity or resistance to apoptosis are not clear. The p53 responses (i.e. specific target genes) may be different in different cell types or the effects of p53 activation may be different depending on other cell-specific factors that

contribute to the apoptotic decision. In MEFs, E1A leads to activation of E2F-dependent transcription and, as discussed previously, many apoptotic targets may be under E2F control, potentially lowering the threshold of additional apoptotic signaling needed for apoptosis. Also, in the E1A-transduced MEF system, p53 levels are increased, priming the cell for a strong response to p53 activation, and cell cycle arrest function is disabled. Binding factors may also play a role in conferring specific p53 responses, and different cell types will differ in the expression of such cofactors. For example, ASPP1 and 2 are proteins the bind p53 and enhance the DNA binding activity and transactivation ability on promoters of apoptotic but not cell cycle arrest genes (Samuels-Lev et al., 2001). Thus, the levels of p53 induced following stress, the recruitment of specific p53 protein partners, and the sensitivity of a specific cell to apoptosis all may contribute to determining specific outcomes of p53 activation.

Downstream p53 effectors- cell cycle arrest

The CDK inhibitor *p21* is a critical p53 target gene for cell cycle arrest function of p53, as irradiation-induced G1 arrest is compromised in cells lacking p21 (Brugarolas et al., 1995). In addition to the very well established role of p53 in a G1 checkpoint, p53 also has been implicated in regulation of G2/M progression. In a colorectal cancer cell line, p53 induces the expression of 14-3-3sigma, which may cause G2 arrest by binding and sequestering CDC2 and cyclin B1 in the cytoplasm, although the mechanism of 14-3-3sigma-mediated arrest is debated (Chan et al., 1999; Hermeking et al., 1997)). p53 also induces the expression of Gadd45 (Kastan et al., 1992) which has been implicated in conferring G2 arrest (Wang et al., 1999). In MEFs, similar to loss of p53, the absence of

Gadd45 leads to genomic instability (Hollander et al., 1999). p53 induces the expression of Reprimo that can induce arrest in G2 (Ohki et al., 2000).

Downstream p53 effectors- apoptosis

p53 confers transcriptional activation of many proapoptotic targets, depending on the extent of stress, and cell type. These p53 targets include many members that regulate the mitochondrial pathway to apoptosis such as *Apaf1, Bax, Puma* and *Noxa* (Vousden, 2000). Many apoptotic stimuli impinge on the mitochondria, causing changes in the permeability of the mitochondrial membrane. The mitochondria release proapoptotic factors such as cytochrome C, a required cofactor that acts in concert with Apaf1 to activate various downstream caspases (Danial and Korsmeyer, 2004). The caspases are the main effectors of the apoptotic program, cleaving many cellular proteins in the early steps in dismantling of the cell. Control of mitochondrial release of proapoptotic proteins is partly determined by the ratio of pro- to anti-apoptotic members of the Bcl2 family. One current model is that proapoptotic members Bax and Bak form pores that insert in the mitochondrial membrane and control the release of cyctochrome C (Danial and Korsmeyer, 2004) . Pro-apoptotic BH3-only proteins including NOXA and PUMA, which are p53 target genes, promote this process. These BH3 domain containing proteins are thought to bind and inhibit pro-survival Bcl2-like proteins (Huang and Strasser, 2000). While much remains to be understood about the biochemical action of the pro- vs. anti-apoptotic Bcl2 family members, it is clear that the balance of these molecules in the cell is critical, and p53 regulation of this process can tip the balance towards apoptosis. p53 also induces the expression of genes such as *PTEN* that negatively regulates cell

survival (Stambolic et al., 2001) and p53 induces proapoptotic targets such as *Perp* whose mechanism of action remains to be determined (Attardi et al., 2000). Consistent with the idea that transactivation of these genes is important for apoptosis, inactivation of many of these target genes including *Bax, Perp, Apaf1, Puma* and *Noxa* impairs p53-dependent apoptosis (Chong et al., 2000; Ihrie et al., 2003; Jeffers et al., 2003; McCurrach et al., 1997; Villunger et al., 2003).

Consistent with the idea that p53 transactivation is critical, ES cells with mutation in two residues critical for transactivation were generated (p53 QS mutants), and p53-dependent thymocyte apoptosis was completely abolished in QS cells (Chao et al., 2000). However, there have been indications that p53-dependent transcription may not be essential for p53-dependent apoptosis in every cellular context. Study of germline transmission of the same QS mutant led to phenotypes different from p53-/- animals. Apoptosis downstream of DNA damage was impaired, but apoptosis downstream of hypoxia was surprisingly retained (Johnson et al., 2005). Furthermore, in contrast to the viability of *p53-/-* animals, embryonic lethality resulted with homozygous QS mutation (Johnson et al., 2005). This supports the idea that transactivation of p53 target genes may not always be essential for apoptosis. Other data has supported the idea of p53 apoptotic function independent of transactivation. For example, in immortalized cells expressing a temperature sensitive p53 allele, p53-dependent apoptosis occurred even with inhibition of new protein or RNA synthesis (Caelles et al., 1994). More recently, a small fraction of p53 has been found localized to the mitochondria (Chipuk et al., 2003; Marchenko et al., 2000; Mihara et al., 2003). In thymocytes mitochondrial p53 was found to form

complexes with anti-apoptotic Bcl2 family members (Mihara et al., 2003). Also, addition of recombinant p53 triggered cytochrome C release from isolated mitochondria, although the biological importance of these observations still remains to be determined. While p53 transactivation function is clearly important for apoptotic function, other activities of p53 independent of transactivation may also contribute.

p53 inactivation in mice

Mouse models have been invaluable for the study of p53 function as a tumor suppressor. Homozygous inactivation of *p53* (Clarke et al., 1993; Donehower et al., 1992; Jacks et al., 1994) resulted in mice that were viable but tumor prone. Homozygous *p53* null animals rapidly developed thymic lymphomas and sarcomas, clearly establishing a tumor suppressor role for p53. *p53* heterozygotes were also tumor prone, exhibiting a longer tumor latency and tumors often exhibited loss of heterozygosity (LOH) (Donehower et al., 1992; Harvey et al., 1993; Jacks et al., 1994). The tumor spectrum included sarcoma, osteosarcoma and lymphoma. Decreased *p53* dosage could also lead to tumor formation, as a careful analyses of the status of the wild-type allele in tumors revealed that the wild-type allele is sometimes both retained and functional in tumors from *p53* heterozygous animals (Venkatachalam et al., 1998). Complete inactivation of *p53* did not mirror the tumor spectrum observed with Li-Fraumeni syndrome. To more accurately model Li-Fraumeni syndrome, point mutant mice were recently generated. *p53R172H/-* and *p53R270H/-* mice were more prone to carcinoma development compared to *p53-/-* mice (Olive et al., 2004). Also, *p53R172/+* and *p53R270H/+* cells exhibited dominant negative effects and an altered tumor spectrum (Lang et al., 2004; Olive et al., 2004).

p53 has pleiomorphic functions, and the nature of the activity of p53 important for p53 tumor suppressor function is not clear. Mouse models suggest that p53 has broad roles in tumor suppression that differ depending on cellular context. It had been thought that mediation of apoptosis was the critical tumor suppressive role of p53. Supporting this idea, in the choroid plexus epithelium, inactivation of Rb-family function using a truncated form of Large-T antigen (Tg121) (which targets the Rb family) led to apoptosis. With additional mutation of *p53*, apoptosis was suppressed and tumorigenesis resulted (Symonds et al., 1994). However, emerging evidence suggests that differing functions of p53 may be critical in tumor suppression depending on the cellular context. Study of a murine point-mutant p53 (Arg172Pro) in which apoptotic function was inactivated, but cell cycle arrest function at least partially retained has led to some insights into this (Liu et al., 2004). The early, aneuploid, T-cell lymphomas to which *p53*-/- mice succumb did not result, and thus, the apoptotic function of p53 was not essential for suppressing these tumors. However, other lymphomas and sarcomas, which were largely diploid, emerged in these animals, and in these it is possible that apoptotic function of p53 may be important for tumor suppression. These experiments suggested that p53-dependent cell cycle arrest and/or maintenance of genomic stability was important for p53 tumor suppressor function in T-cells. While *p53*-/- MEFs rapidly become aneuploid in culture, the Arg172Pro point mutant MEFs were diploid (Liu et al., 2004). These data also raise the possibility that different mutations of p53 may lead to differences in tumor spectrum, depending on whether any residual p53 function is

retained. Such refined mouse models have been helpful both for better mimicking human tumors that inactivate p53, as well as for dissecting many aspects of p53 biology.

This thesis describes the use of mouse genetics to gain insights into the in vivo functions of two tumor suppressor proteins that are central players in cancer. These genes are quite different; in the case of *Rb*, gene knockout studies reveal critical roles for *Rb* in development, while *p53* function is largely dispensible for development but plays a role in the response to stress. The role of pRB in development is likely to be highly related to pRB function as a tumor suppressor. Indeed, the human tissue most sensitive to pRB loss leading to tumorigenesis is the retina, in which tumors have developmental origins. This thesis describes work to gain insight into pRB function in development and tumor suppression. Initial experiments described in Chapter 2 address questions raised by prior analyses of the *Rb* knockout embryo. The *Rb* mutant embryo has been a system used extensively in genetic analyses of pathways downstream of *Rb* loss. To interpret these genetic data, cell autonomous vs. non-cell autonomous effects of gene deletion in a given tissue are important to understand. To investigate the possibility that signals from defective blood cell development contribute to the *Rb*-/- CNS phenotype, we studied embryos lacking pRB in the CNS on a background of normal blood cell development. This was important in clarifying the involvement of secondary signals required for cell death in the embryo. It also allowed study of the role of pRB in the development of tissues at later stages of development than could be previously examined. In chapter 3, I discuss the results of a focused analysis of pRB function in the retina. This work has helped to elucidate the role of pRB in retinal biology, with very different effects of pRB

deletion found in different cell types. In addition, breedable mouse models of retinoblastoma were developed to understand the genesis and progression of these tumors and p130 function as a tumor suppressor demonstrated.

A detailed understanding of pathways leading to p53 modifications and the purpose of modifications that occur in vivo is critical to understanding the role of p53 in tumor suppression. *p53* is the most frequently mutated tumor suppressor gene and the mutational status of *p53* can dictate the response to therapy (Lowe et al., 1994a). A fine level of understanding of p53 activation may also help us understand how to activate p53 function in tumor cells in which p53 is intact, as well as better understand the important pathways that converge on p53. Chapter 4 describes work to understand the importance of a single p53 modification site, which had surprisingly strong, but cell context-specific effects in regulation of p53-dependent responses and tumorigenesis.

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Chapter 2

Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system

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The author performed all experiments described in Figures 1 through 7. Julien Sage generated the *Rb lox/lox* animals, Andreas Trumpp generated the *NesCre1* transgenic, Denise Crowley cut many histological sections for this study. Experiments were done in the lab of Tyler Jacks.

Abstract

Targeted disruption of the retinoblastoma gene in mice leads to embryonic lethality in midgestation accompanied by defective erythropoiesis. Rb-/- embryos also exhibit inappropriate cell cycle activity and apoptosis in the central nervous system (CNS), peripheral nervous system (PNS) and ocular lens. Loss of p53 can prevent the apoptosis in the CNS and lens; however, the specific signals leading to p53 activation have not been determined. Here we test the hypothesis that hypoxia downstream of defective erythropoiesis in Rb null embryos contributes to p53-dependent apoptosis. We show evidence of hypoxia in CNS tissue from Rb-/- embryos. The Cre-loxP system was then used to generate embryos in which Rb was deleted in the CNS, PNS and lens, in the presence of normal erythropoiesis. In contrast to the massive CNS apoptosis in Rb null embryos at E13.5, conditional mutants did not have elevated apoptosis in this tissue. There was still significant apoptosis in the PNS and lens, however. Rb-/- cells in the CNS, PNS and lens underwent inappropriate S-phase entry in the conditional mutants at E13.5. By E18.5, conditional mutants had increased brain size and weight as well as defects in skeletal muscle development. These data support a model in which hypoxia is a necessary co-factor in the death of CNS neurons in the developing Rb mutant embryo.

Introduction

The retinoblastoma tumor suppressor is an important regulator of the cell cycle, differentiation, and apoptotic death (reviewed in Gallie et al (1999), Lipinski et al, 1999, Weinberg, 1995). Germline mutations in the RB gene predispose individuals to bilateral retinoblastoma as well as osteosarcoma (reviewed in Gallie, 1999). Somatic inactivation of RB contributes to the development of these tumor types as well as prostate, breast, lung and bladder cancer. Disruption of "retinoblastoma pathway" function through direct RB mutation or mutation of upstream regulators of pRB such as CDK4, or $p16^{INK4a}$ is thought to occur in the vast majority of human cancers (Weinberg, 1995).

Studies involving the targeted disruption of Rb in mice have provided significant insight into the function of pRB in normal development and tumor suppression. Mice heterozygous for a Rb mutation develop pituitary and thyroid tumors, which exhibit loss of the remaining wild-type Rb allele (Hu et al, 1994, Williams et al, 1994). Homozygosity for an Rb mutation causes embryonic lethality near embryonic day 14.5 (E14.5) (Clarke et al, 1992, Jacks et al, 1992, Lee et al, 1992). Rb null embryos are pale and exhibit defects in fetal liver erythropoiesis. Absence of pRb function also causes dramatic defects in the lens, central nervous system (CNS) and peripheral nervous systems (PNS). In these tissues, both inappropriate S-phase entry, and high levels of apoptosis are evident (Clarke et al, 1992, Jacks et al, 1992, Lee et al, 1992, Lee et al, 1994, Morgenbesser et al, 1994).

Extensive analyses of the molecular pathways contributing to these phenotypes have been carried out using compound mutant analysis. pRB binds to members of the E2F family of transcription factors to regulate G1 to S-phase progression (reviewed in

Dyson, 1999). Compound mutant analysis involving deletion of both Rb and either E2f1 or E2f3 supports a critical role of these transcription factors in Rb function (Tsai et al, 1998, Ziebold et al, 2001). Mutation of both Rb and E2f1 or E2f3 led to reduced levels of the inappropriate S-phase entry and apoptosis in the CNS and lens. The erythropoietic defect was also partially rescued in these compound mutants, extending embryo survival until near E17.5. Compound mutant analysis has also implicated the p53 tumor suppressor in apoptosis in the Rb-deficient CNS and lens but not PNS (MacLeod et al, 1996, Morgenbesser et al, 1994). Loss of p53 specifically inhibited apoptosis in the setting of Rb deficiency; inappropriate S-phase and embryonic lethality were not affected. The specific signals that mediate p53 activation in the Rb-/- embryos have not been determined, although it is known that deregulated E2f activity can cause p53 activation in other systems. In cell culture, E2f1 overexpression leads to proliferation and apoptosis that is partially p53-dependent (Kowalik et al, 1998, Qin et al, 1994, Wu et al, 1994). One possible mediator of p53 activation downstream of E2f members is the E2f target ARF, which regulates p53 by inhibiting MDM2 (Pomerantz et al, 1998) and is induced in mouse embryonic fibroblasts upon E2f1 overexpression (Bates et al, 1998). However, loss of Arf does not significantly inhibit the CNS or lens apoptosis in Rb mutant embryos (Tsai et al, 2002).

The analysis of chimeric animals composed of both wild-type and Rb null cells has demonstrated that both the development of erythroid cells and the death of Rb-/- neurons can be rescued (Lipinski et al, 2001, Maandag et al, 1994, Williams et al, 1994). With respect to inhibition of apoptosis in the CNS, it is possible that neighboring cells provide survival signals in the form of a secreted factor or perhaps involving cell-cell

contacts that allow nearby Rb-deficient cells to survive (Lipinski et al, 2001). An alternative possibility is that the survival of CNS neurons is due to the absence of a pro-apoptotic factor normally present in germline Rb-/- embryos. Specifically, the normal development of the hematopoietic system in these chimeras may relieve hypoxic stress on the embryo, thus eliminating a critical signal for apoptosis.

The cause of the defect in erythropoiesis in Rb-/- embryos is not known, although it has been proposed that the Rb deficient fetal liver may not provide an environment supportive of erythrocyte development. Rb mutant fetal livers are hypocellular, have high levels of apoptosis, and produce a deficit of mature, enucleated erythrocytes. However, in Rb+/+:Rb-/- chimeras mature enucleated red blood cells derived from Rb-deficient cells are present (Maandag et al, 1994, Williams et al, 1994). Also, fetal livers from Rb null embryos can reconstitute a lethally-irradiated host (Hu et al, 1997), again suggesting that the environment in which the erythrocytes develop is a critical factor. Given that the apoptosis in the Rb null CNS is non-cell autonomous, we hypothesized that hypoxia downstream of defective erythropoiesis may contribute to p53-dependent apoptosis in the Rb-null CNS.

Upregulation of p53 protein in neurons has been demonstrated upon ischemic injury in vivo (Li et al, 1994, Yamaguchi et al, 2002) and upon hypoxia treatment of neurons cultured in vitro (Banasiak et al, 1998, Zhu et al, 2002). Also, neurons cultured from p53-deficient animals showed resistance to hypoxia-mediated cell death (Halterman et al, 1999), supporting the idea that hypoxia can lead to p53-dependent apoptosis in neurons. It is also possible that hypoxia could contribute to p53-dependent apoptosis in the Rb-/- lens, or p53-independent apoptosis in the Rb-/- PNS. In the Rb-/- PNS, the

mechanism of apoptosis appears mechanistically distinct from that in the CNS. Apoptosis in this tissue is not affected on a p53 null background (MacLeod et al, 1996), and loss of E2F1 or E2F3 only partially reduces the PNS apoptosis in the absence of Rb function (Tsai et al, 1998, Ziebold et al, 2001).

To determine if hypoxia due to the erythropoietic defect was required for apoptosis in the Rb null embryos, we used a conditional gene targeting approach to remove Rb from the CNS, PNS and lens while maintaining normal erythropoiesis. This was carried out using the Cre-loxP system in which Cre expression was driven by regulatory elements from the rat nestin gene (Fan et al, 2001, Trumpp et al, 1999, Bates et al, 1999). In this system, Cre is expressed efficiently in nervous system progenitor cells as well as in other tissues leading to deletion of Rb in the CNS, PNS and lens. As such, we were able to examine the fate of Rb-/- cells in these tissues in the presence of an intact hematopoietic system.

Results

Expression of hypoxia-inducible genes in Rb-/- CNS

In order to determine whether the CNS in Rb-/- embryos may be under hypoxic stress that could contribute to p53-dependent apoptosis, we examined the expression of the hypoxia-inducible genes vascular endothelial growth factor (VEGF) and lactate dehydrogenase A (LDH-A) in microdissected brain tissue using Northern blotting. As shown in Figure 1, increased expression of VEGF and LDH-A was observed in CNS tissue of E13.5 Rb-/- embryos compared to controls. Our observation of upregulation of these genes provides indirect evidence that these animals may be under hypoxic stress.

Given that hypoxia would be expected to be secondary to the defective erythropoiesis in the Rb-/-embryo, we sought to conditionally eliminate Rb in the nervous system and determine the function of Rb mutant cells.

Generation of conditional Rb mutants

To remove Rb conditionally, the endogenous Rb locus was targeted to introduce loxP sites surrounding exon 3. Generation of mice with both alleles containing loxP sites flanking Rb exon 3 (termed $Rb^{2lox/2lox}$) will be described elsewhere (J.S and T.J. in preparation). We bred $Rb^{2lox/2lox}$ mice to Nes-Cre1 transgenic mice in which Cre expression is driven by regulatory elements of the rat nestin gene (Bates et al, 1999, Fan et al, 2001, Trumpp et al, 1999) (Fig. 2a). The expression of Cre in this strain has been shown to begin prior to E9, resulting in almost complete Cre-mediated recombination in the midgestation brain as well as in the germline (Bates et al, 1999, Fan et al, 2001). Cre-mediation recombination has been observed less efficiently in other tissues, including skeletal muscle (Fan et al, 2001). All crosses were done with the male bearing the Cre transgene due to previously-reported imprinting effects with the Nes-Cre1 transgene (Bates et al, 1999, Trumpp et al, 1999). Male $Rb^{2lox/+}$ carrying the Cre transgene were crossed to $Rb^{2lox/2lox}$ females. Due to Cre expression in the germline, conditional Rb mutants arising from this cross carried the Nes-Cre1 transgene and had the genotype $Rb^{1lox/2lox}$ at the Rb locus. Littermates with genotype $Rb^{1lox/2lox}$ that lacked the Cre transgene served as controls. Southern blot analysis demonstrated recombination in the E13.5 brain of conditional mutants bearing the Nes-Cre1 transgene (Fig 2b). We confirmed by Western blot that pRB was indeed absent from whole-embryo brain at

E13.5 in conditional mutants with the Cre transgene, but levels of pRB in the liver and torso of Cre-expressing animals were similar to controls (Fig 2c). We also documented removal of pRB in the PNS, by performing Western blots on microdissected dorsal root ganglia from E13.5 embryos. Nestin is normally expressed in the developing mouse lens (51), and microdissection of E13.5 lenses from conditional mutants and subsequent western blot analysis showed decreased pRB levels in conditional mutants expressing the Nes-Cre1 transgene (Fig 2c). Thus, this strategy allowed us to delete Rb from several tissues in which apoptosis and inappropriate S-phase occurs in germline Rb-/- embryos.

Normal erythropoietic development in conditional mutant animals

As shown in Figure 2d, conditional Rb mutants had normal liver cellularity and levels of apoptosis compared to controls. They were also normally superficially vascularized, in contrast to the pale appearance of Rb-/- embryos (not shown). As demonstrated previously, peripheral blood smears from Rb-/- embryos have predominantly nucleated erythrocytes (93.6+/-3.1% nucleated n=4). Rb conditional mutants, however, exhibit normal production of enucleated erythrocytes (Fig. 2E). 57.4+/-6.1% of erythrocytes from conditional mutants were nucleated (n=4), which is similar to the frequency seen in controls (55.1+/-4.5% nucleated n=3). Importantly, while overexpression of VEGF and LDH-A was observed in germline Rb-/- CNS tissue, levels of these hypoxia-inducible genes were normal in conditional mutant tissue, providing indirect evidence that the conditional mutant embryos were not under hypoxic stress (Fig. 1). These data indicated that erythropoiesis was normal in conditional mutants and allowed us to focus on the phenotypic consequences of loss of Rb function in normoxic CNS, PNS and lens.

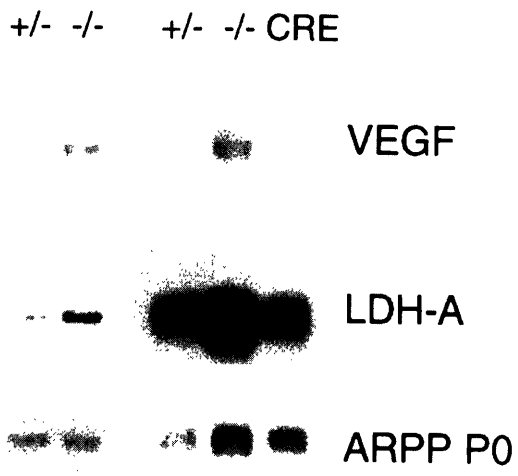
+/- -/- +/- -/- CRE
VEGF
LDH-A
ARPP P0

Figure1. Expression of hypoxia-inducible genes in Rb-/- CNS. Northern blotting showing expression levels of the hypoxia-inducible genes VEGF and LDH-A in Rb+/-, Rb-/- and conditional Rb mutant (CRE) E13.5 whole brain tissue. ARPP P0 was used as a loading control.

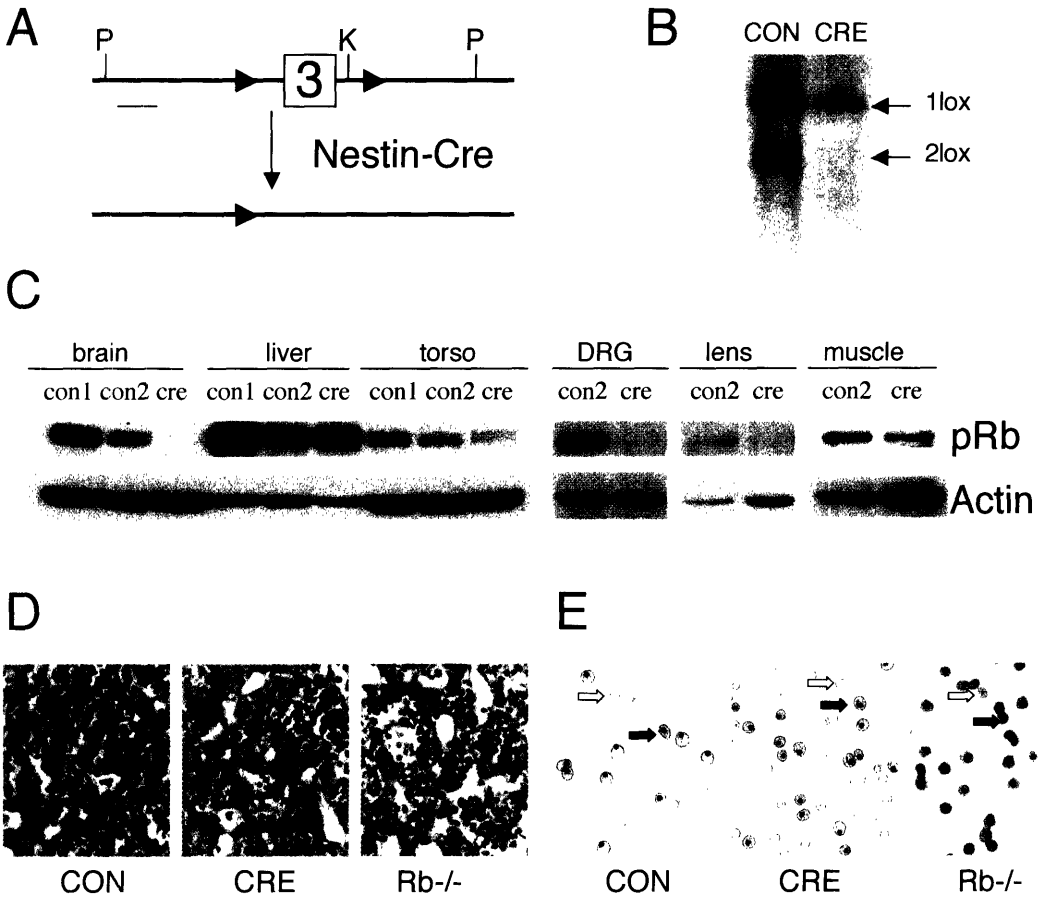
A
P
K
P
3
Nestin-Cre
B
CON CRE
1lox
2lox
C
brain
liver
torso
DRG
lens
muscle
con1 con2 cre
con1 con2 cre
con1 con2 cre
con2 cre
con2 cre
con2 cre
pRb
Actin
D
CON
CRE
Rb-/-
E
CON
CRE
Rb-/-

Figure 2. Conditional removal of Rb in developing nervous system and lens with rescue of erythropoietic defect. A) Schematic representation of Rb conditional knockout allele. Cre-mediated recombination removes exon 3 of Rb in tissues expressing Cre from regulatory elements of the rat nestin gene. P, Pst1 site, K, Kpn site B) Southern Blot of genomic DNA from E13.5 whole brain with the genotype $Rb^{1lox/2lox}$ lacking the cre transgene (CON), or bearing the Nes-Cre1 transgene (CRE). Genomic DNA was digested with Pst1 and the Kpn isoscizhomer Acc65I. The bottom 6.5kb band is the Rb^{2lox} unrecombined allele, while the larger 9kb band is the recombined Rb^{1lox} allele. C) Western blot showing tissue specific loss of Rb conditional mutants carrying the Nes-Cre1 transgene. Tissue is from E13.5 embryos except for skeletal muscle, which was from E18.5 embryos. CON1 refers to control embryos with 2 copies of the Rb gene product (genotype $Rb^{2lox/+}$) while CON2 refers to embryos with 1 copy of the Rb gene product (genotype $Rb^{1lox/2lox}$). CRE refers to conditional mutants (genotype $Rb^{1lox/2lox}$) carrying the Nes-Cre1 transgene. Torso is the remainder of embryo lacking the head, liver and heart. DRG, dorsal root ganglia D) Hematoxylin and eosin stained sagittal section showing normal liver hematopoiesis in conditional mutant animals, while Rb-/- livers show decreased cellularity and pycnotic nuclei. E) Peripheral blood smear from E13.5 control, conditional mutant or Rb null embryos. Note normal ratio of enucleated definitive erythrocytes (open arrows) to nucleated erythrocytes (closed arrows) in conditional mutant smear, while Rb -/- smears have very few enucleated erythrocytes. See text for quantitation.

Rescue of midgestation apoptosis but not S-phase entry in CNS

We first determined the phenotype of conditional mutant animals at E13.5, which is the time point that Rb null embryos normally exhibit high levels of CNS apoptosis. Strikingly, levels of apoptosis in the conditional mutant embryos were similar to controls and dramatically lower than in the Rb-/- CNS (Figs. 3 and 4). The data shown are for the hindbrain where apoptosis in Rb null embryos is particularly high; however, equivalent suppression of apoptosis was observed throughout the conditional mutant brain (data not shown). These data suggest that the death of Rb-/- neurons may be dependent on a hypoxic state induced by defective erythropoiesis. In contrast to the dramatic suppression of CNS apoptosis, the levels of ectopic S-phase determined by BrdU incorporation away from the ventricular zone was similar in conditional mutants compared to Rb null embryos (Figs. 3 and 4).

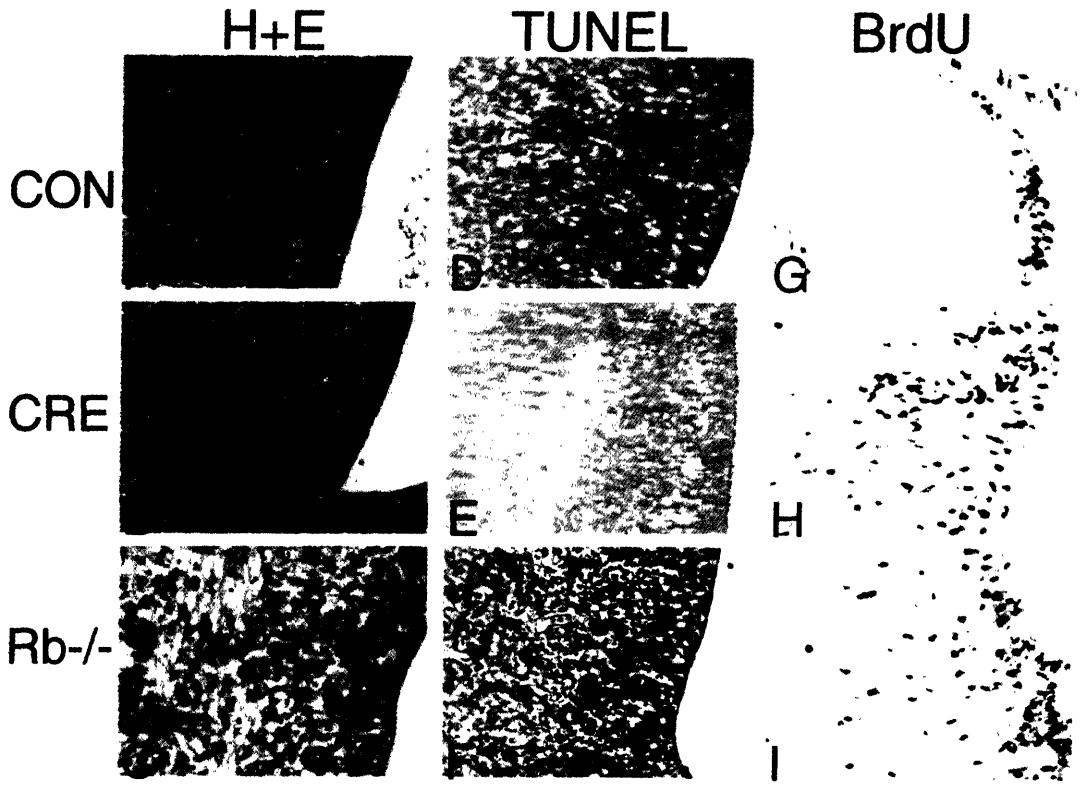
H+E
TUNEL
BrdU
CON
CRE
Rb-/-
D
E
G
H
I

Figure 3. Apoptosis and S-phase entry in mid-sagittal sections of hindbrain and 4th ventricle from control, conditional Rb mutant and Rb null E13.5 embryos. H+E stain (A,B,C) and TUNEL staining (D,E,F) showing apoptotic cells at 40X magnification. Rb null (C,F) hindbrain has numerous darkly staining apoptotic bodies, while apoptosis in conditional mutant (B,E) hindbrain is similar to controls (A,D) . BrdU analysis of S-phase entry at 40X magnification (G, H, I). In controls (G) , BrdU positive cells are restricted to the ventricular zone, while in conditional mutant (H) or Rb null (I) sections, extensive ectopic BrdU positive cells are seen in the intermediate zone.

Apoptosis in conditional mutant PNS and lens.

We next determined the phenotype of the conditional Rb mutant PNS and lens. In contrast to the CNS, levels of apoptosis in the conditional mutant PNS were similar to those seen in Rb-/- embryos (Figs. 4 and 5). The data shown are for the dorsal root ganglia (DRG), but similar results were obtained from the trigeminal ganglia (data not shown). The DRG neurons of conditional mutants underwent inappropriate S-phase entry comparable to Rb-/- embryos (Figs. 4 and 5). In the normal lens, epithelial cells proliferate at the outer edge of the tissue and migrate posteriorly before exiting the cell cycle. Following cell cycle exit, they migrate into the interior of the lens and differentiate into lens fiber cells. BrdU positive cells are not normally present in the lens fiber cell compartment (Fig. 6). However, in both Rb conditional mutant and Rb null lenses, ectopic BrdU positive cells were readily apparent (Fig 6). Apoptosis in the lens fiber cell compartment in Rb null E13.5 lenses has been shown to be p53-dependent (Morgenberrer et al, 1994). Interestingly, in contrast to control lens sections, conditional mutant lenses had significant levels of apoptosis. Apoptosis in the conditional mutant lens was comparable, though quantitatively lower than apoptosis observed in the Rb null embryo lens (Figs. 4 and 6). Thus, in the lens and PNS, loss of Rb appears to be sufficient to cause apoptosis even in the presence of a normal hematopoietic system. These data underscore the mechanistic differences in the apoptotic programs in the Rb-deficient CNS, PNS and lens.

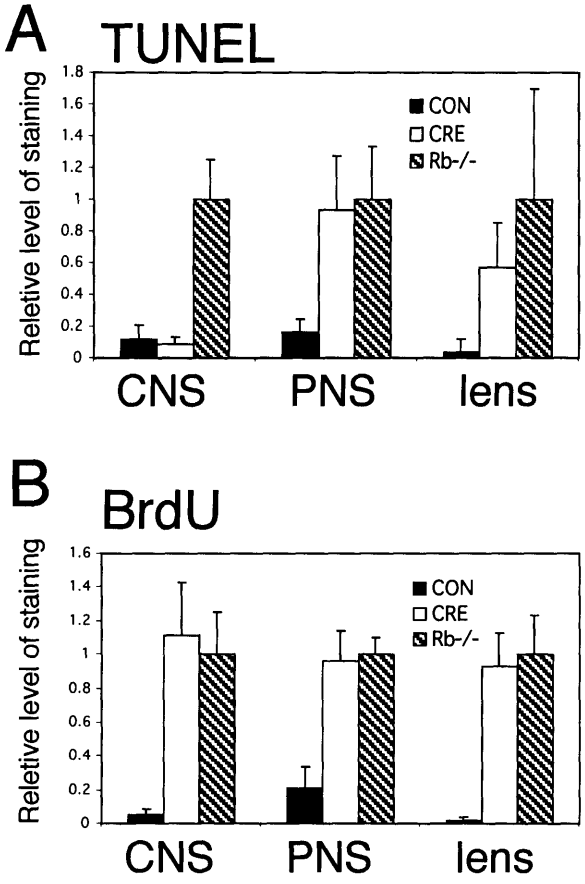
A
TUNEL
Reletive level of staining
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CON
CRE
Rb-/-
CNS
PNS
lens
B
BrdU
Reletive level of staining
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CON
CRE
Rb-/-
CNS
PNS
lens

Figure 4. Quantitation of apoptosis and S-phase entry in Rb mutant and conditional mutant embryos. A) Conditional mutation of Rb leads to apoptosis in the PNS and lens but not in the CNS. Apoptotic cells were quantified as number of TUNEL positive nuclei per area of tissue measured in the hindbrain adjacent to the 4^{th} ventricle, dorsal root ganglia and ocular lens. Apoptosis is expressed relative to the amount seen in Rb null embryos which was set to 1.0. Standard deviation is indicated by error bars. B) Extent of inappropriate S-phase entry is similar between Rb null embryos and conditional mutants in the lens, CNS and PNS. For the CNS, ectopic S-phase was quantified as BrdU positive cells outside of the ventricular zone and quantified per area of tissue measured. For the PNS, overall S-phase in the dorsal root ganglia was quantified per area of tissue measured. For the lens, BrdU positive cells in the lens fiber compartment were quantified per area of tissue measured. S-phase entry is expressed relative to the amount seen in Rb null embryos which was set to 1.0. Standard deviation is indicated by error bars. All data are from five to ten groups of embryos of a given genotype.

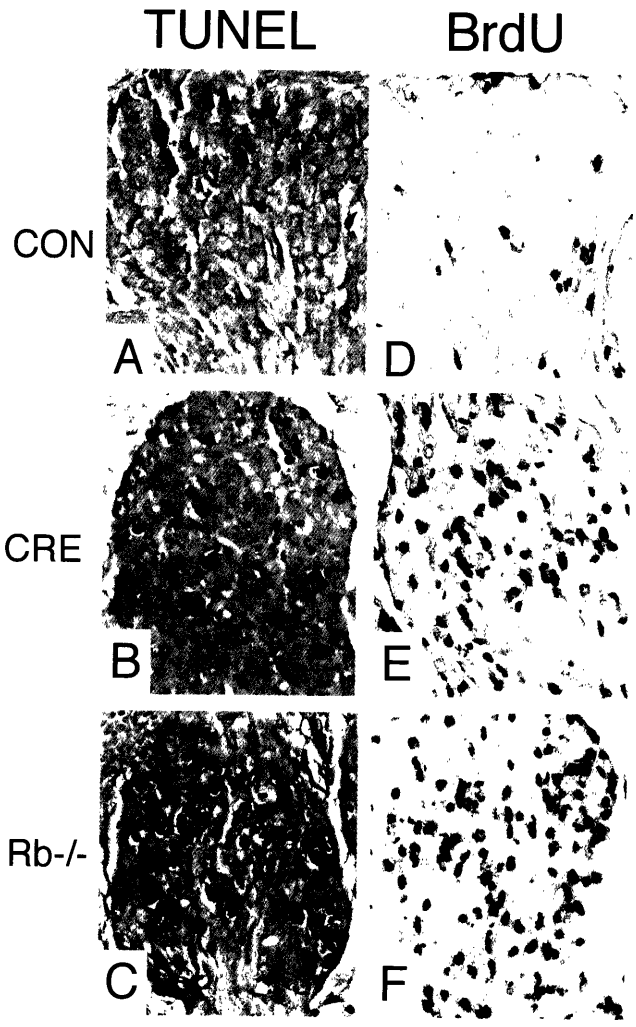
TUNEL
BrdU
CON
CRE
Rb-/-
A
B
C
D
E
F

Figure 5. Apoptosis and S-phase entry in sections of dorsal root ganglia from control, conditional Rb mutant and Rb null E13.5 embryos. (A to C) TUNEL staining of E13.5 dorsal root ganglia at 40X magnification. Note the darkly staining apoptotic cells seen at increased levels in the conditional mutant (B) and Rb null (C) sections. BrdU staining of E13.5 dorsal root ganglia at 40X magnification shows extensive S-phase activity in both conditional mutant (E) and Rb null (F) ganglia.

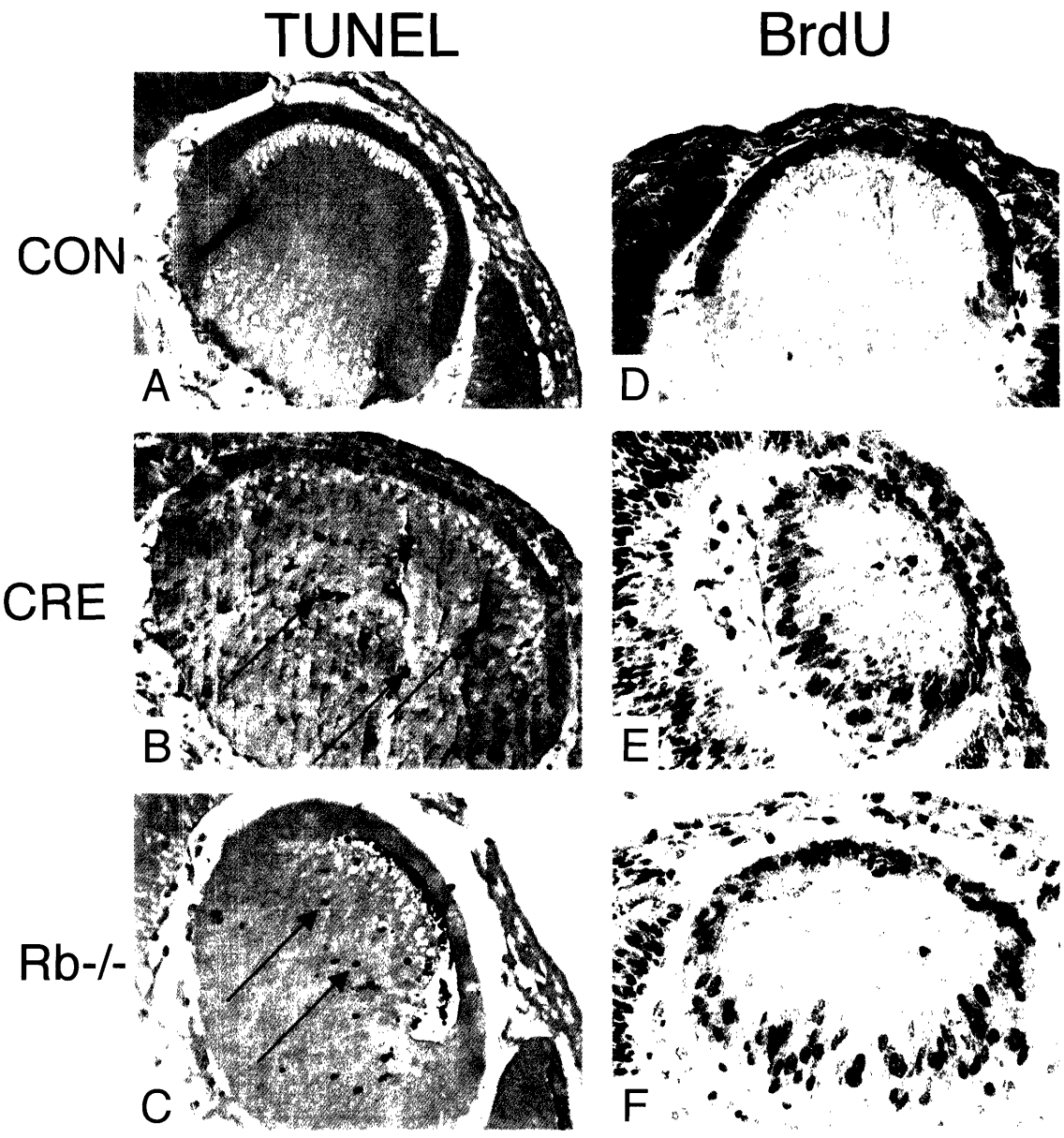
TUNEL
BrdU
CON
CRE
Rb-/-
A
B
C
D
E
F

FIG. 6. Apoptosis and S-phase entry in transverse sections of ocular lens from control, conditional Rb mutant, and Rb-null E13.5 embryos. TUNEL staining for apoptosis at a magnification of 40 (A to C) or 100 (D to F) demonstrates numerous darkly stained apoptotic nuclei (arrows) in both conditional-mutant (B and E), and Rb-null lens (C and F) sections, but not in controls (A). BrdU staining at a magnification of 40 (G to I) demonstrates ectopic S-phase entry in the lens fiber cell compartment in lenses from both conditional mutants (H) and Rb-null (I) embryos, whereas BrdU-positive cells are restricted from this compartment in control sections (G).

Rescue of development with skeletal muscle defects in Rb conditional mutants

To determine if the normal erythroid development would affect the survival of Rb conditional mutants, we collected embryos throughout gestation and determined the frequency of different genotypes recovered. Conditional Rb mutant embryos were found in Mendelian ratios throughout embryonic development, including those collected at E18.5 (data not shown). At E18.5, conditional mutant embryos were not visibly pale, but could be clearly distinguished from their littermates by their hunched appearance (Fig 7a). Conditional-mutant embryos were observed to be born alive but died within 30 min following birth. We were interested in the consequence of aberrant S-phase activity in the E18.5 brain, due to the observed increased S-phase entry without apoptosis in earlier developmental stages. At E18.5, brains from conditional Rb mutant animals were visibly larger than littermate controls (Fig. 7b). Recently, a telencephalon specific Rb knockout was generated, which had shown cell survival in the telencephalon, and increased cortical size (Ferguson et al., 2002). In the whole-brain Rb conditional mutants we have described, effects of Rb loss on brain size were not restricted to the telencephalon, as most of the brain appeared enlarged (Fig. 7B and data not shown). Measurements of brain weights (Fig. 7C) indicated that conditional-mutant brains weighed 27% more than controls ($P = 0.001$). There was no evidence of hydrocephalus in these mutant brains (not shown). We are currently investigating the effects of Rb absence on regional brain development and cortical lamination in these conditional-mutant brains.

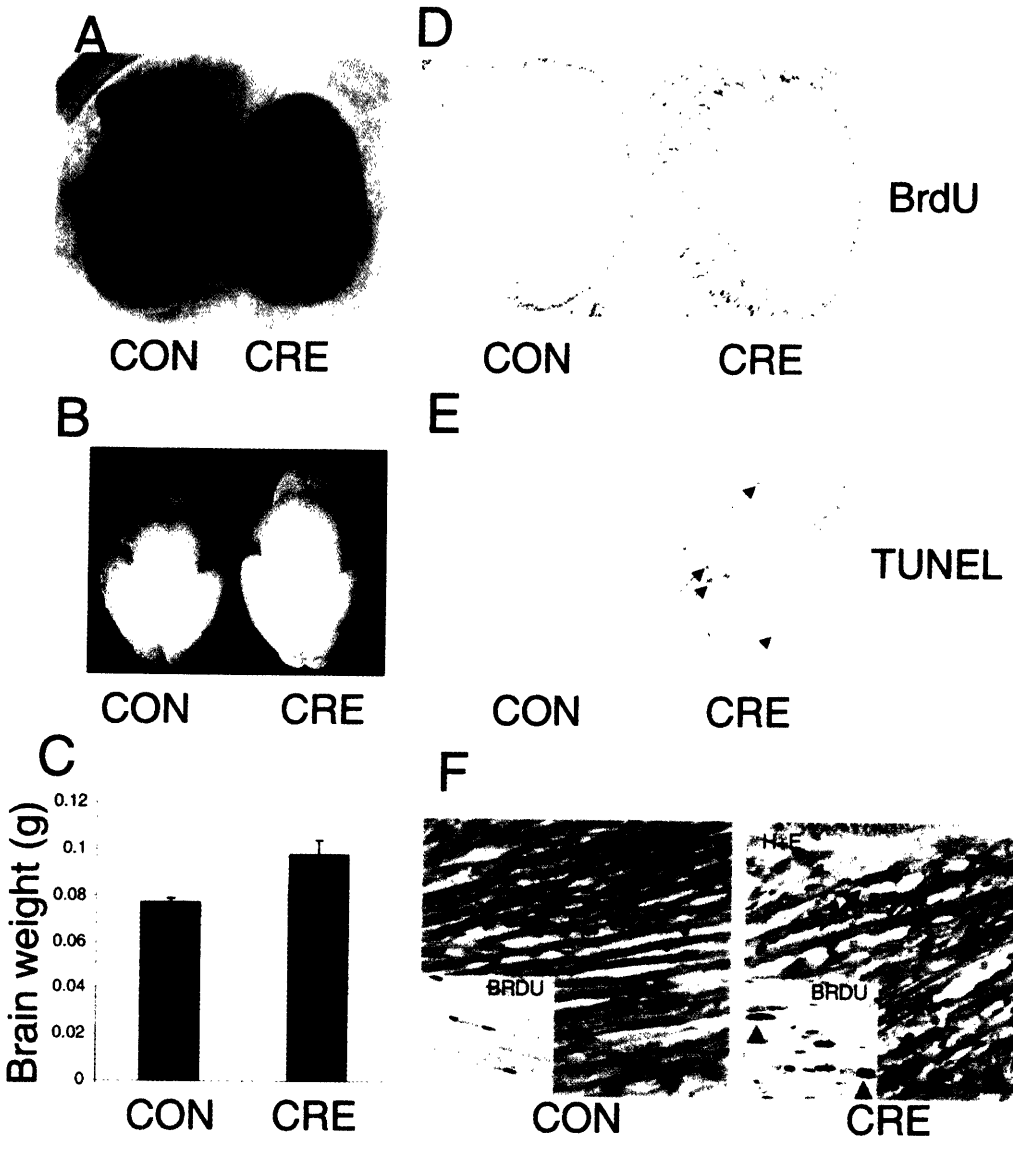
A
CON
CRE
D
BrdU
CON
CRE
B
CON
CRE
E
TUNEL
CON
CRE
C
Brain weight (g)
0.12
0.1
0.08
0.06
0.04
0.02
0
CON
CRE
F
BRDU
BRDU
CON
CRE

FIG. 7. Phenotype of conditional-mutant embryos at E18.5. (A) Picture of E18.5 control (CON) and conditional-mutant (CRE) animals. Note the hunched appearance of the conditional-mutant embryo. (B) Conditional-mutant brains are visibly larger than those of littermate controls. (C) Measurement of brain weights indicates that conditional-mutant brains weigh 27% more than littermate controls (t test; P 0.001). (D) BrdU analysis of ocular lens demonstrates continued ectopic S-phase entry in the conditional-mutant (CRE). (E) TUNEL analysis of lens shows high levels of apoptosis (arrows) in conditional-mutant lens (CRE). (F) Hematoxylin-eosin (HE)-stained sections of axial skeletal muscle from E18.5 control (CON) and conditional-mutant (CRE) animals demonstrating defective muscle differentiation in conditional mutants. Note the enlarged atypical nuclei (arrows) and abnormal rows of adjacent nuclei apparent in the conditional-mutant sections. The inset shows BrdU analysis demonstrating active DNA-synthesis in abnormally large nuclei in conditional mutant (arrowheads).

At E18.5, phenotypes were also observed in the ocular lens and skeletal muscle. In the lens, BrdU analysis indicated that cells continued to enter S-phase ectopically (Fig. 7D). TUNEL analysis indicated that conditional mutants continued to show high levels of apoptosis (Fig. 7E). Importantly, partial recombination in skeletal muscle has been reported with the Nes-Cre1 transgene used here (Fan et al, 2001), and we also observed decreased pRB levels in conditional mutant skeletal muscle compared to controls (Fig. 2c). In late stages of development in conditional embryos, impairment of skeletal muscle differentiation was evident (Fig. 7f). Areas of conditional Rb mutant skeletal muscle at E18.5 had strikingly abnormal large nuclei, and the arrangement of myotubes appeared diffuse (Fig 7f). BrdU analysis demonstrated that some of the abnormally large nuclei apparent in these mutants exhibited inappropriate S-phase activity. (Fig. 7f). We also observed abnormal rows of adjacent nuclei in conditional-mutant myotubes that were not apparent in controls. We have not determined the cause of lethality of the conditional-mutant animals. Perinatal lethality of Rb mutants with development rescued through expression of low levels of Rb with an Rb transgene (Zacksenhaus et al, 1996) or with Id2 deficiency (Lasorella et al, 2000) had been ascribed to muscle defects affecting respiration. However, given the pertubation in brain development observed in these mutants here, it is also possible that deletion of Rb in the brain may impair nervous system control of respiration or other vital nervous system functions.

Discussion

In this study we have demonstrated that the CNS tissue of Rb-/- embryos shows increased expression of the hypoxia-inducible genes LDH and VEGF and, using a conditional deletion strategy, we have shown that Rb-/- CNS neurons are spared from apoptosis in an embryo whose erythroid development is normal. Thus, we conclude that hypoxia secondary to the erythroid defect is a likely co-factor in the death of these Rb-/- cells. Importantly, the cell cycle defects associated with Rb mutation in the CNS were still evident in the conditional mutants, supporting the contention that these are cell autonomous. While this work was in preparation, another group reported that conditional mutation of Rb in the telencephalon region of the brain led to inappropriate S-phase entry of Rb-mutant cells without high levels of apoptosis (Ferguson et al, 2002). The reason for the strikingly different phenotypes observed in the telencephelon of germline Rb mutants and telencephalon-specific conditional Rb mutants was not determined. Our study extends the phenotype reported in the conditional mutant telencephalon and demonstrates cell survival throughout the E13.5 brain, with increased brain size observed at E18.5. In addition, we provide evidence that the germline mutant Rb brain is under hypoxic stress that is relieved with conditional mutation of Rb. Our findings in the CNS and the results of (Ferguson et al, 2002) raised the possibility that apoptosis in other tissues in germline Rb mutant embryos may also be dependent on signals such as hypoxia carried by the blood. The tissue pattern of Rb deletion using the Nes-Cre1 transgene allowed us to determine if deletion of Rb in the PNS and lens would lead to cell death in the presence of normal hematopoiesis. Consistent with previous work that has uncovered

differences in the apoptotic program among different cell types in the Rb-/- embryo, we found significant cell death in the PNS and lens of conditional Rb mutants.

We have previously reported that in E13.5 chimeric embryos composed of both wild-type and Rb null cells, there was suppression of CNS apoptosis (Lipinski et al, 2001) without rescue of inappropriate S-phase entry. There were a number of possible explanations for these findings. For example, in such chimeras, absence of Rb in the cells of the developing CNS might directly trigger the apoptotic program (perhaps as a consequence of cell cycle dysfunction), but this program may be repressed by survival signals stemming from wild-type cells. These survival signals could have originated from cells in the CNS itself in the form of secreted or cell-surface factors. Alternatively, such survival signals could derive from wild-type cells outside of the CNS and be transmitted to the CNS from a distance via the blood. The data from chimeras could also be explained by the existence of pro-apoptotic factors produced by Rb-/- cells. In this scenario, Rb-/- cells in the CNS or originating outside of the CNS may produce a pro-apoptotic factor that acts on CNS neurons but in Rb+/+:-/- chimeras this factor might be diluted below a critical threshold needed for induction of apoptosis.

By using the Nes-Cre1 transgene, we were able to remove Rb throughout the E13.5 brain (Fig. 2c) and observed absence of increased apoptosis in this tissue. The widespread deletion of Rb in the CNS allows us to exclude the possibility that survival signals were sent from wild-type cells originating in this tissue. Also, we can now conclude that CNS apoptosis in Rb-/- embryos is due to the production of a pro-apoptotic factor, and we propose that this signal is hypoxia. Hypoxia has been demonstrated to be an inducer of p53 in neurons and other cell types (Banasiak et al, 1998, Graeber et al,

1994, Li et al, 1994, Yamaguchi et al, 2002), and there is clear severe anemia and defective development of erythrocytes in Rb-/- embryos. Indeed, the anemia is thought to be responsible for the death of midgestation Rb-/- embryos, and we have demonstrated that conditional Rb mutants with normal erythropoiesis develop until birth. There is also indirect evidence of hypoxia in Rb-/- embryos with the overexpression of VEGF and LDH-A observed only in the germline Rb mutant but not in the conditional mutant CNS. We support the hypothesis that the defects in erythropoiesis causes hypoxia leading to upregulation of VEGF and LDH-A, as well as apoptosis. However it is possible that other unknown cell extrinsic signals could cause induction of VEGF and LDH and it is still unresolved whether the presence or absence of other signals present in developing Rb-/- blood system might contribute to the death of CNS neurons.

Another unresolved issue is whether Rb-deficient neurons may be sensitized to hypoxia-mediated apoptosis. Rb-/- cells in the CNS show inappropriate S-phase entry that conceivably could make these cells sensitive to hypoxia-induced apoptosis. In cell culture, hypoxia treatment was demonstrated to lead to p53 induction specifically in an S-phase enriched cell population (Hammond et al, 2002). The mechanism for p53 activation in S-phase was proposed to be via a hypoxia-induced replication arrest and subsequent activation of the ATR kinase upstream of p53 (Hammond et al, 2002). While primary cells in culture have been shown to be resistant to hypoxia-mediated apoptosis, oncogenic transformation of such cells, involving disruption of pRb family function through E1A expression, can confer strong sensitivity to hypoxia-mediated, p53-dependent apoptosis (Graeber et al, 1996). It is possible that loss of cell cycle control in Rb mutants may sensitize these cells to hypoxia-mediated apoptosis. Emerging evidence

also suggests that in some systems pRB plays an anti-apoptotic role that may be broader than effects of Rb on the cell cycle. For example, exposure of cultured post-mitotic neurons to DNA damaging agents led to rapid phosphorylation of pRB by cyclin-dependent kinases prior to apoptosis (Park et al, 1998, Park et al, 2000). Importantly, introduction of a phosphorylation-resistant Rb mutant conferred protection against DNA-damage-induced apoptosis suggesting that phosphorylation of pRB was important for the progression of apoptosis (Liu et al, 2001, Park et al, 2000). It has also been proposed that caspase cleavage and subsequent degradation of pRB early in apoptosis is an important event in the execution of apoptosis (Chen et al, 1997, Janicke et al, 1996, Tan et al, 1997). In cultured neurons and MEFs, introduction of a cleavage-resistant pRB mutant also conferred strong protection against apoptosis (Boutillier et al, 2000, Tan et al, 1997). pRB-mediated suppression of apoptotic factors may be lost with pRB cleavage or when Rb is constitutively absent through germline mutation. The pro-apoptotic signals repressed by pRB are not known; however in theory such signals could be enhanced in Rb-/- embryos and sensitize neurons to apoptosis. Similarly, Rb-/- MEFs exhibit sensitization to apoptosis induced by DNA damage or growth factor withdrawal (Almasan et al, 1995).

The phenotype of Rb mutant embryos has been used in extensive genetic analyses that have helped define the genetic pathways converging on apoptosis upon Rb loss. In the CNS and lens, E2f1, E2f3, p53 and Apaf1 have all been shown to be required for apoptosis (Guo et al, 2001, Macleod et al, 1996, Tsai et al, 1998, Ziebold et al, 2001). The data presented here may necessitate reinterpretation of these results. For example, given the rescue of both the inappropriate S-phase and apoptosis in the CNS when both

E2f1 and Rb were mutated together, it was concluded that inappropriate S-phase through deregulation of E2f activity led to p53 activation (Tsai et al, 1998). Links between deregulated E2F and p53-dependent apoptosis have been found in both cell culture systems and transgenic animals. For example, choroid plexus expression of a fragment of SV40 large T that targets Rb family members but not p53 leads to proliferation and p53-dependent apoptosis, which were both suppressed on an E2f1-deficient background (Pan et al, 1998). In cell culture, overexpression of E2f1 leads to upregulation of Arf, and p53-dependent apoptosis (Bates et al, 1998, Kowalik et al, 1998, Qin et al, 1994, Wu et al, 1994). We subsequently searched for E2F targets that may directly mediate p53 activation in Rb null embryos and found that the E2F target ARF was not required for p53-dependent apoptosis in the CNS (Tsai et al, 2002). In the CNS, it now appears that the rescue of apoptosis observed with Rb/E2f1 compound mutation may be explained by indirect effects on erythropoiesis. Compound mutation of Rb and E2F1 led to a partial though incomplete rescue of erythropoiesis at E13.5 (Tsai et al, 1998). However, in contrast to the conditional mutants described here, by E17.0 compound Rb/E2f1 animals appeared anemic and died, indicating that rescue of erythropoiesis was incomplete. We propose that the partial rescue of erythropoiesis in Rb/E2f1 mutants may have been sufficient to reduce the hypoxic stress needed for apoptosis in Rb-/- neurons.

Interestingly, the complete rescue of CNS apoptosis by Rb/E2f1 compound mutation was accompanied by only partial rescue of apoptosis in the PNS (Tsai et al, 1998). Similarly, it was striking that even though apoptosis in the CNS occurred at normal levels in conditional mutants, there was a clear increase in apoptosis in the conditional mutant PNS. The pathway leading to apoptosis in the Rb-/- PNS is largely

undefined. It is known that levels of apoptosis are roughly correlated with levels of inappropriate S-phase entry, p53 is dispensable for this death, and caspase3 is required in this system (Macleod et al, 1996, Simpson et al, 2001). Our present study further confirms that the pathways to apoptosis in the Rb-/- CNS and PNS are functionally distinct. Levels of apoptosis were similar to germline Rb mutants suggesting that in contrast to CNS neurons, conditional Rb deficient PNS neurons were not sensitized to apoptosis. The finding that neurons in the PNS were not sensitized to apoptosis induced by the cell-extrinsic signals in germ line Rb mutants indicates that that the apoptotic machinery may be profoundly different in the developing Rb-/- CNS versus PNS. Interestingly, mutation of caspase 3 did not inhibit the CNS apoptosis in germ line Rb-/- embryos but completely inhibited the PNS apoptosis (Simpson et al, 2001). Differences in the importance and amounts of various apoptotic factors such as caspase 3 across different cell types could very well control the life or death of the cell in response to hypoxia or other apoptotic stresses. Therefore, future work elucidating the apoptotic programs activated in response to Rb loss in the different cell types could help us define the basis for these cell type differences. Note that because the Nes-Cre1 transgenic mouse is not specific to the nervous system, we cannot rule out the possibility that deletion of Rb in an unknown tissue compartment could contribute to PNS apoptosis. It will be important to determine the genetics of this p53-independent apoptosis, which appears to be cell autonomous. Because tumors that have mutated p53 are often resistant to chemotherapy, elucidating mechanisms to induce apoptosis in proliferating cells independent of p53 function may be relevant to chemotherapeutic development.

Our findings in the lens further illustrate that the pathway leading to apoptosis upon Rb loss differs in different cell types. p53-dependent apoptosis in Rb conditional mutant lens still occurred with rescue of the erythropoietic defect indicating that the pathway upstream of p53 is different in the Rb null lens vs. CNS. In the Rb deficient lens, previous findings that E2f1 or E2f3 loss led to a rescue of apoptosis points to a role for E2f1 and E2f3 in p53-activation that is more direct than the pathway leading to p53 in the CNS. The findings here related to the Rb-deficient lens agree with our previous observations that adult chimeras composed of Rb-/- and wild-type cells had high levels of apoptosis in the lenses (Williams et al, 2004), and we also observed high levels of apoptosis in late gestation E18.5 conditional mutant lenses (Fig.7e). Disruption of Rb family function in the lens by expression of the HPV E7 oncoprotein has also been shown to cause lens cell proliferation and apoptosis (Pan et al, 1994). Apoptosis in this setting was also suppressed by removal of p53 function through E6 expression.

Hypoxia in tumors that have mutated the Rb pathway may indeed be a source of selective pressure for loss of p53 as a means to evade apoptosis. Importantly, hypoxia treatment of oncogenically transformed MEFs in vitro led to selection for cells with mutated p53 (Graeber et al, 1996). In tumors, apoptosis was seen in regions of hypoxia, while p53 null tumors were resistant, suggesting that tumors select for loss of p53 to become resistant to hypoxia-induced apoptosis (Graeber et al, 1996). It may be interesting to search for genetic links between hypoxia and p53-dependent apoptosis in embryos lacking Rb. For example, HIF1α has been implicated in p53 signaling downstream of hypoxia (An et al, 1998, Carmeliet et al, 1998), and it would be interesting to know if the pathway to apoptosis in the Rb deficient CNS is HIF1α-

dependent. It will also be important to determine if kinases upstream of p53 activation are involved in signaling to p53 in this system. Dissecting the pathways leading to CNS apoptosis in Rb-/- mouse embryos may help us to elucidate the pathways that connect hypoxia to p53 activation, and selection for p53 mutation in human tumors.

Methods

Mice and generation of embryos

Male Nes-Cre1 mice were initially crossed to $Rb^{2lox/2lox}$ mice. Male offspring expressing Cre were crossed to $Rb^{2lox/2lox}$ females. Details on PCR genotyping reactions are available upon request. The morning of plug detection was taken as E0.5, and embryos were collected throughout development. Embryos were dissected from the mother, the yolk sac was collected for genotyping and embryos were fixed overnight in 3.7% formaldehyde in PBS. Embryos were processed to paraffin, embedded and 4μm sections were cut.

Northern Blotting

Whole brain tissue was quickly dissected from E13.5 embryos and frozen on dry ice. Tissue was homogenized in Trizol Reagent (Invitrogen) and total RNA was isolated following the manufacturers instructions. Northern blotting with 10μg total RNA was performed using standard methods. VEGF and LDH-A cDNA's were used as probes. VEGF cDNA was a gift of Volker Haase and LDH-A cDNA was obtained through RT-PCR. Probe Labeling was performed using the Prime-It II Random Primer labeling kit (Stratagene) and hybridization was performed using ExpressHyb solution (Clontech).

Southern Blotting

Genomic DNA was isolated from E13.5 whole brains following digestion of tissue with Proteinase K and extraction with phenol/chloroform. Genomic DNA was digested with

Pst1 and Acc65i, run on a 0.8% agarose gel, transferred to a nylon membrane (Hybond N+, Amersham) and hybridized with a P^{32} labeled internal probe as described (Jacks et al, 1992).

Western blotting

Whole embryo brain, dorsal root ganglia and ocular lens were dissected from E13.5 embryos and tissue was frozen on dry ice. For dorsal root ganglia microdissection, the E13.5 spinal cord was separated out and ganglia subsequently were removed from the spinal cord and pooled. Skeletal muscle was dissected from E18.5 embryos. Tissue was lysed in 100mM NaCl, 100mM Tris pH8, 1%NP-40 and Complete Protease Inhibitor Cocktail (Roche). Protein was separated by SDS-Page and transferred to a PVDF membrane (Immobilon). Blots were first probed with an antibody to pRB (Pharmingen, 1/1000). Blots were then stripped, and reprobed with an antibody to actin (Santa Cruz, 1/1000). HRP conjugated secondary antibodies (Jackson Immunochemicals) were used at a 1/5000 dilution. Enhanced chemiluminescence (ECL+, Amersham) was used for signal detection before exposing blots to film.

Apoptosis and BrdU staining in embryos

For BrdU analysis, pregnant females were injected intraperitoneally with BrdU (Sigma) at 30μg/gm body weight 1 hour before animal sacrifice and embryo dissection. Staining for BrdU was performed as described (Tsai et al, 2002). For analysis of apoptosis, terminal-deoxynucleotidyl transferase (TdT)-mediated dUTP-end labeling (TUNEL) was used (Gavrielli et al, 1992). Paraffin sections were rehydrated, treated with proteinase K

and incubated in TUNEL mixture including biotin labeled dUTP (Roche), and rTdT (Invitrogen). Detection of incorporation of biotin labeled dUTP was done using the ABC (Vector labs) and detection with DAB (Vector Labs).

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Chapter 3

Cell-type specific effects of Rb deletion in the murine retina

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The author performed all immunostaining, TUNEL and BrdU analyses with technical assistance from Teresa Kim and Dennis Ho. The author also performed all Western and Northern blots. Meg MacLaughlin performed the staining for LacZ activity in Supp. Fig.1. Experiments were done in the lab of Tyler Jacks

Abstract

Certain cells of the human retina are extremely sensitive to loss of function of the retinoblastoma tumor suppressor gene *RB*. Retinoblastomas develop early in life and at high frequency in individuals heterozygous for a germline *RB* mutation, and sporadic retinoblastomas invariably have somatic mutation in the *RB* gene. In contrast, retinoblastomas do not develop in *Rb*+/- mice. While retinoblastoma is thought to have developmental origins, the function of *Rb* in retinal development has not been fully characterized. Here we study the role of *Rb* in normal retinal development and in retinoblastoma using conditional *Rb* mutations in the mouse. In late embryogenesis, *Rb*-deficient retinas exhibited ectopic S-phase and high levels of p53-independent apoptosis, particularly in the differentiating retinal ganglion cell layer. During post-natal retinal development loss of *Rb* led to more widespread retinal apoptosis, and adults showed loss of rod photoreceptors and bipolar cells. Conditional *Rb* mutation in the retina did not result in retinoblastoma formation even in a *p53*-mutant background. However, on a *p107*- or *p130*-deficient background, *Rb* mutation in the retina causes retinal dysplasia or retinoblastoma.

Introduction

The *RB* gene was the first known tumor suppressor gene identified (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). In humans, inheritance of a mutant allele of *RB* leads to retinoblastoma at about a 90% frequency, with loss of the remaining wild-type allele thought to be a rate-limiting step in tumorigenesis. *RB* is also mutated in sporadic cancers of the brain, breast, bladder, lung and bone (Horowitz et al. 1990). Moreover, mutation of some component of the "*RB* pathway," such as loss of $p16^{INK4a}$ or amplification of upstream *RB* regulators *cyclin D* or *CDK4/6* is thought to occur in the majority of human cancers. Germline *RB*+/- humans are at some increased risk for tumors other than retinoblastoma, such as osteosarcomas (Eng et al. 1993); however, no tissue is as exquisitely sensitive to *RB* loss as the retina. The reason for this tissue sensitivity is unknown.

The product of the *RB* gene, pRB, is a nuclear phosphoprotein that is an important regulator of the cell cycle (Weinberg 1995). pRB forms complexes with members of the E2F family of transcription factors to repress transcription of genes important for S-phase entry and progression. Interaction of pRB with chromatin remodeling enzymes such as HDAC appears to contribute to gene repression. Phosphorylation of pRB by cyclin-dependent kinases leads to dissociation of pRB from E2Fs and release of pRB-mediated repression. In addition to regulating S-phase entry, *RB* also regulates apoptosis and terminal differentiation and has been implicated in still other cellular processes (Lipinski and Jacks 1999; DiCiommo et al. 2000; Classon and Harlow 2002; Chau and Wang 2003).

In contrast to most human tumors, retinoblastomas usually arise during the first few years of life. Retinoblastomas have also been found in the developing human fetus in utero (Salim et al. 1998). Therefore, elucidating the role of *RB* in normal retinal development should help to explain the origins of this tumor. The adult retina consists of seven cell types, including six neuronal cell types and one type of glial cell, arranged into three nuclear layers. The outer nuclear layer contains rod and cone photoreceptor cell bodies. The inner nuclear layer includes bipolar, amacrine and horizontal neuron cell bodies as well as the cell bodies of the single glial cell type, the Muller glia. The innermost retinal ganglion cell layer contains both ganglion cells and displaced amacrine cells. The cells in the adult retina develop from multipotent progenitor cells in a birth order that changes as development proceeds (Turner and Cepko 1987; Turner et al. 1990). Both intrinsic properties of progenitor cells and responses to extrinsic cues dictate retinal cell fate (Cepko et al. 1996; Marquardt and Gruss 2002). Retinoblastomas have been proposed to derive from a primitive neuroectodermal cell (Kyritsis et al. 1984), and evidence of photoreceptor differentiation has been described in some tumors (Bogenmann et al. 1988; Vrabec et al. 1989; Tajima et al. 1994). However, the cell of origin of retinoblastomas has been controversial, and there has been limited examination of markers specific to neuronal retinal cell types other than photoreceptors in human tumor material.

Early attempts to model retinoblastoma in the mouse through germline mutation of *Rb* were not successful. Germline *Rb*+/- animals never develop retinoblastomas, although they do develop pituitary and thyroid tumors at high frequency (Jacks et al. 1992). Germline *Rb* mutant homozygous embryos die in midgestation with defects in

erythropoiesis and placental development (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Wu et al. 2003). Chimeric *Rb*++;/-- mice survive to adulthood, but these mice also do not develop retinoblastomas (Maandag et al. 1994; Williams et al. 1994b). An examination of retinas from chimeric embryos revealed increased apoptosis (Maandag et al. 1994), and retinas from adult chimeras showed a relatively low contribution from *Rb*-/- cells (Maandag et al. 1994; Williams et al. 1994b).

Rb is the founding member of a gene family containing two other members, *p107* and *p130*. These proteins have structural similarities in the so-called A/B pocket region that is important for binding of pRB to E2F family members and to DNA tumor virus oncoproteins such as SV40 T Antigen, human papillomavirus E7 and adenovirus E1A (Weinberg 1995). The three "pocket protein" family members have both overlapping and distinct cellular and developmental functions (Classon and Harlow 2002; Trimarchi and Lees 2002). For example, in contrast to the essential role of *Rb* during development, *p130*-/- or *p107*-/- mice on a similar genetic background survive to adulthood without clear phenotypes (Cobrinik et al. 1996; Lee et al. 1996).

Human retinoblastomas have not been found to carry mutations in *p107* or *p130*, although absence of p130 expression has been found in a subset of tumors (Bellan et al. 2002). Also, the chromosomal arm harboring *RB2/p130* undergoes frequent alteration in human retinoblastomas (Mairal et al. 2000; Chen et al. 2001; Herzog et al. 2001; Lillington et al. 2003). In the mouse, while *Rb*-/-;+/+ chimeras do not develop retinoblastoma, chimeras composed of cells lacking both *Rb* and *p107* developed retinoblastomas with characteristics of amacrine cells (Robanus-Maandag et al. 1998). Embryonic retinas from these chimeras exhibited dysplasia with high levels of apoptosis.

Loss of both copies of *p107* in an *Rb*+/- background also causes retinal dysplasia in adults (Lee et al. 1996). These data indicate that *Rb*-deficient retinas have tumorigenic potential that is realized with *p107* mutation and suggest that *p107* can compensate for *Rb* loss in the murine retina. A potential role for *p130* in compensating for retinal *Rb* loss has not been reported.

Understanding the role of *Rb* in the development of the different retinal cell types is critical to understanding both the cause of retinoblastoma and the retinal cell type susceptible to transformation. Loss of *Rb* in chimeric embryo retinas has been associated with increased apoptosis, and it has been proposed that mutation to evade apoptosis may be required for retinoblastoma development (Gallie et al. 1999). However, the response of specific cell types in the retina to *RB/Rb* deletion has not been fully examined. Here we use conditional mutation to delete *Rb* in early retinal progenitors in the mouse. We characterize the cell-type specific effects of *Rb* loss in the development of the retina and report the cell-type characteristics of a novel murine model of retinoblastoma that develops in the absence of retinal *Rb* and *p130.*

Results

We have previously described the use of a conditional loss-of-function allele of *Rb* (Rb^{lox}) combined with the expression of Cre-recombinase from the *nestin* promoter (*NesCre1*) (MacPherson et al. 2003). In this system, *Rb* deletion occurs in the developing nervous system and other tissues; the resulting embryos survive gestation and die at birth (MacPherson et al. 2003). We and others have observed a different degree of Cre-mediated recombination from the *NesCre1* transgene depending on whether the transgene

is inherited from the mother or the father, presumably due to imprinting effects (Fan et al. 2001). Paternally-inherited *NesCre1* gives significantly more extensive and complete Cre-mediated recombination. The initial experiments described here were performed from *Rb* conditional embryos with paternally-inherited *NesCre1* (designated *NesCre1(p)*) in order to address the effects of *Rb* deficiency on retinal development. To characterize *NesCre1(p)* transgene activity in the retina, these animals were crossed to a reporter strain that expresses the *LacZ* gene following Cre-mediated deletion of a lox-STOP-lox cassette (Soriano, 1999). Widespread *NesCre1(p)*-driven transgene activity was evident in the optic vesicle from which the retina derives at E9.5 (Supplemental Figure 1a). X-gal staining of adult retinas revealed complete recombination throughout the retina when *NesCre1* is paternally inherited (Supplemental Figure 1 b,c). Consistent with evidence of early and widespread retinal transgene activity from *LacZ* reporter gene analyses, paternal transmission of *NesCre1*–resulted in complete absence of pRB on a $Rb^{lox/lox}$ background in the retina at E13.5 (Figure 1a).

Cell Cycle defects in the *Rb*-deficient retina

Loss of *Rb* in the nervous system has been reported to cause cell cycle defects (Macleod et al. 1996; Ferguson et al. 2002; MacPherson et al. 2003). Therefore, we investigated cell cycle control in *NesCre1(p)* conditional *Rb* mutant retinas. In control retinas, mitosis occurs in the neurepithelium immediately adjacent to the retinal pigment epithelium (RPE). Migration away from this zone occurs during G1, S and G2, and nuclei return to the outer surface for the next mitosis. pRB is highly expressed in the differentiating retinal ganglion cell layer at E18.5 (Jiang et al. 1997). S-phase activity in E18.5 *Rb*-

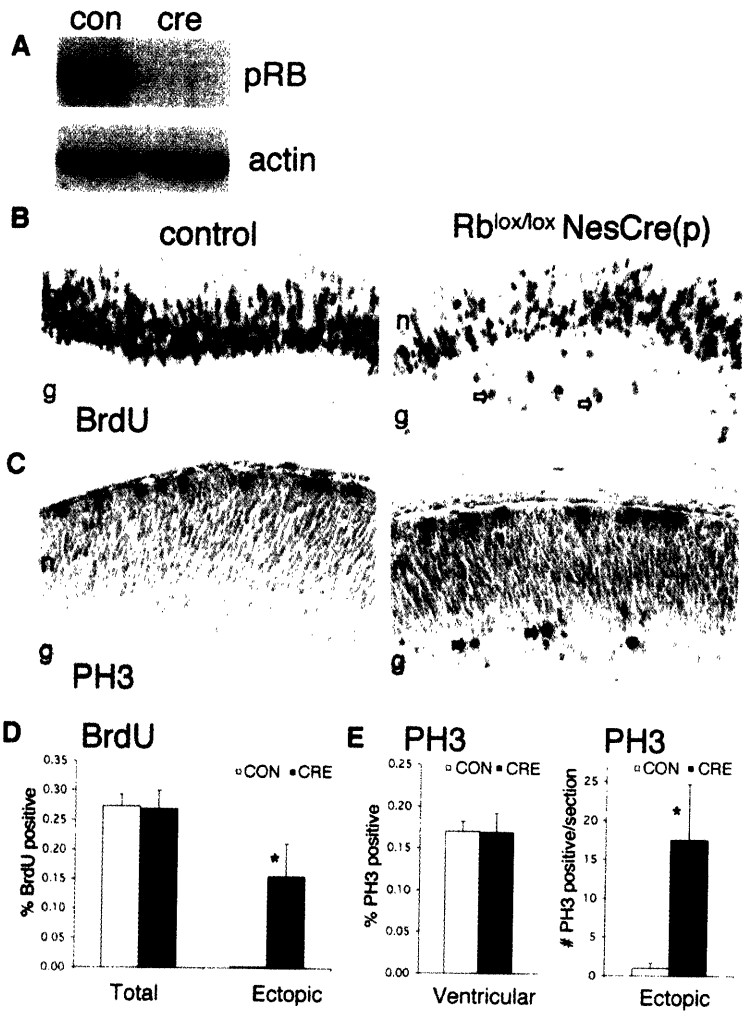
A
con
cre
pRB
actin
B
control
Rb^lox/lox NesCre(p)
n
g
BrdU
C
PH3
D
BrdU
CON CRE
% BrdU positive
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Total
Ectopic
E
PH3
CON CRE
% PH3 positive
0.25
0.20
0.15
0.10
0.05
0.00
Ventricular
PH3
CON CRE
PH3 positive/section
25
20
15
10
5
0
Ectopic

Figure 1. Cell cycle defects in *Rb*-deficient retina. Retinas obtained from paternally inherited *NesCre1(p) Rb* null or control retinas A) Western blot for pRB expression at E13.5 in control or *NesCre1(p) Rb* mutants. B) BrdU analysis for S-phase activity at E18.5 in controls or *NesCre1(p) Rb* mutants. Arrows denote ectopic BrdU-positive cells. C) Phospho-histone H3 (PH3) immunostaining for evidence of mitotic activity at E18.5. Arrows show ectopic mitotic cells in inner layer of *NesCre1(p) Rb* mutants. D) Quantification of overall BrdU positive cells, or ectopic BrdU positive cells. The ratio of BrdU positive to all retinal cells was quantified from the central retina at the level of the optic nerve. Ectopic BrdU-positive cells were those that extend beyond the normal zone of S-phase to the inner ganglion cell layer (*, $p<0.01$; Student's T-test). (n=6) E) Quantification of mitoses along neurepithelium immediately adjacent to RPE (ventricular) or ectopic mitoses outside of the ventricular zone along the entire retina at level of optic nerve (*, $p=0.01$; Student's T-test) (n=6). n, neuroblastic layer; g, ganglion cell layer.

deficient or control retinas was analyzed by BrdU incorporation. In control embryos there is a clear zone of cells that incorporate BrdU, and no BrdU-positive cells were seen in the innermost, retinal ganglion cell layer (Fig 1b,d). In *NesCre1(p)* conditional *Rb* mutants, however, ectopic S-phase activity was prevalent in the retinal ganglion cell layer (Fig. 1b,d). The overall proportion of cells in the retina that were BrdU positive did not differ significantly between *Rb* mutants and controls; however, ectopic S-phase entry was greatly increased in *Rb* mutants (Fig 1b, d). To examine if cells undergoing inappropriate S-phase proceeded to mitosis, we stained for the mitotic marker phospho-histone H3 (PH3). In contrast to controls, where strong PH3 staining was restricted to the retinal ventricular zone in *Rb* mutants ectopic PH3 staining was observed (Fig. 1c,e). Increased PH3 staining was particularly high in the *Rb* mutant retinal ganglion cell layer (Fig. 1c). Cells in this layer normally should have undergone a permanent exit from the cell cycle. Thus, the inappropriate S-phase and mitosis suggests that *Rb*-deficiency causes impairment of cell cycle exit.

Rb-/- retina undergoes p53-independent apoptosis

Histological examination of *NesCre1(p)* conditional *Rb* mutant retinas at E18.5 revealed significant numbers of pyknotic nuclei indicative of apoptosis (not shown). Apoptosis was confirmed using immunostaining for active caspase 3 (Fig 2a,b). Apoptosis was prevalent throughout the retina but was notably higher in the retinal ganglion cell layer. The morphology of cells in the retinal ganglion layer at E18.5 was abnormal with both apoptotic and morphologically aberrant cells throughout. Combined with the data suggesting that *Rb* is required for proper cell cycle exit, the increased apoptosis in this

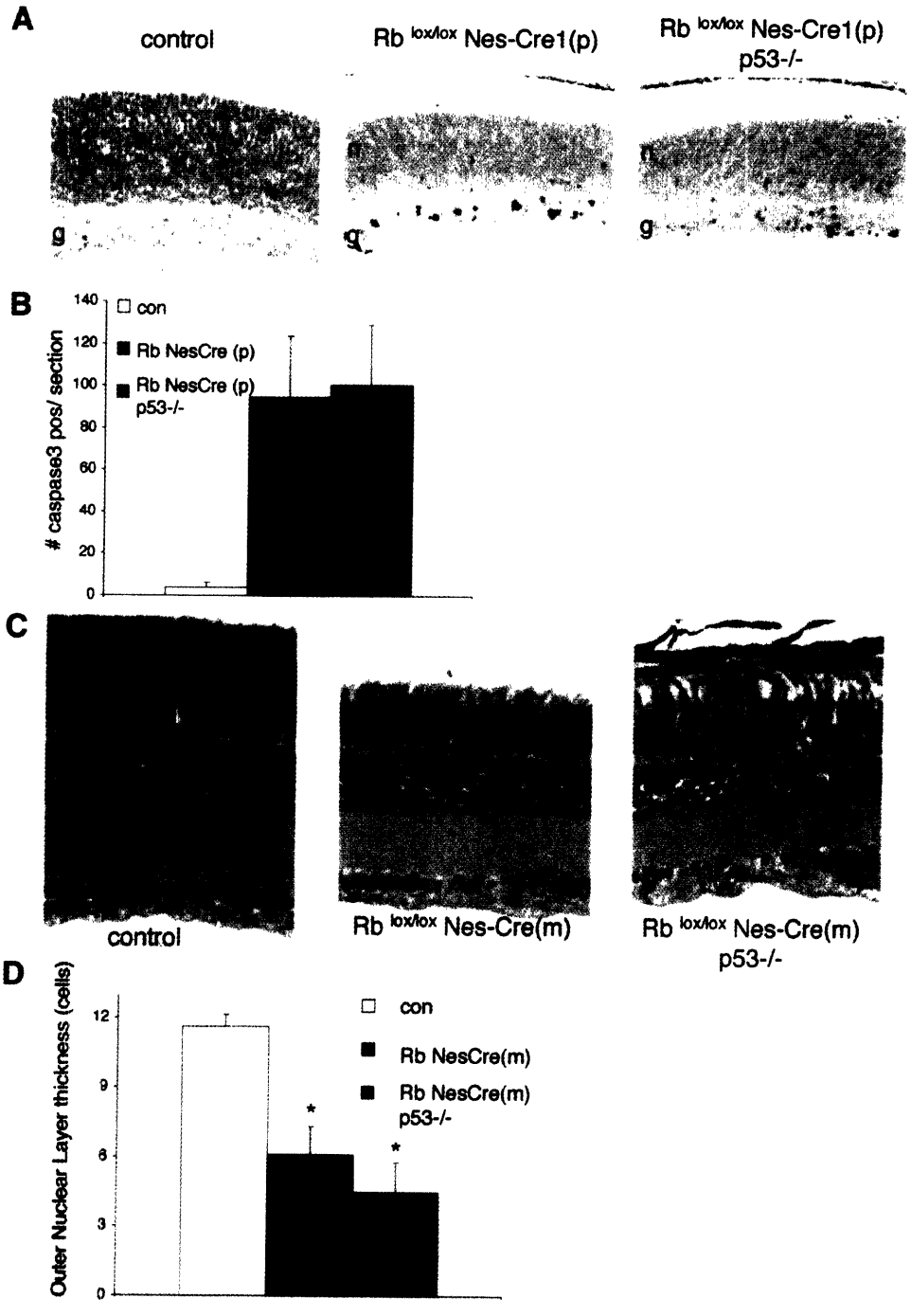
A
control
Rb lox/lox Nes-Cre1(p)
Rb lox/lox Nes-Cre1(p)
p53-/-
g
B
caspase3 pos/ section
140
120
100
80
60
40
20
0
con
Rb NesCre (p)
Rb NesCre (p)
p53-/-
C
control
Rb lox/lox Nes-Cre(m)
Rb lox/lox Nes-Cre(m)
p53-/-
D
Outer Nuclear Layer thickness (cells)
12
9
6
3
0
con
Rb NesCre(m)
Rb NesCre(m)
p53-/-
*
*

Figure 2. p53-independent apoptosis in *Rb*-deficient retina A) Immunostaining for active caspase3 in E18.5 retinas of control, paternally inherited *NesCre1(p) Rb* and *NesCre1(p) Rb;p53-/-* embryos. B) Quantification of Caspase3-positive cells in sections at level of optic nerve (n=6). C) Hematoxylin and Eosin staining of adult *NesCre1 Rb* mosaics and *p53-/- NesCre1 Rb* mosaic adults showing reduced photoreceptor layer. Cre was maternally inherited. D) Quantification of thickness of photoreceptor layer in control (n=4), *Rb NesCre1* (n=8) and *RbNesCre1 p53-/-* adult mosaics (n=4) (*, $p<.01$ vs controls; Student's T-test). n, neuroblastic layer; g, ganglion cell layer; i, inner nuclear layer; o, outer nuclear layer.

cell population would indicate that defects in the coordination of the cell cycle withdrawal and onset of differentiation lead to cell death. However, *Rb* might also inhibit apoptosis more directly (Chau and Wang 2003).

Previous studies have demonstrated *p53*-dependent apoptosis in the CNS of *Rb*-/- embryos and that this death was due to primary defects in the placenta leading to hypoxia (Macleod et al. 1996; Ferguson et al. 2002; de Bruin et al. 2003; MacPherson et al. 2003; Wu et al. 2003). *p53* mutation can also cooperate with *Rb* pathway inhibition to contribute to tumorigenesis by suppressing apoptosis (Howes et al. 1994; Symonds et al. 1994). To determine if the retinal apoptosis was *p53*-dependent, we bred the *Rb Nes-Cre1(p)* mutant animals onto a *p53*-/- background and analyzed retinas at E18.5 as shown in Figure 2. Levels of apoptosis were similar when comparing *Rb* mutants with and without *p53*. Thus, in contrast to previous findings from midgestation *Rb*-/- CNS, we find retinal apoptosis at E18.5 in conditional *Rb* mutants is a more direct consequence of *Rb* deficiency in that it occurs in embryos with normal placentas and normal erythropoiesis and is not dependent on *p53*. While most retinas with *p53* and *Rb* deficiency appeared identical to *Rb* mutants, we noted that one compound *NesCre1(p)* conditional *Rb;p53*-/- mutant that had high levels of apoptosis and also exhibited focal dysplasia in both eyes (not shown). This suggests that effects of *p53* deficiency may vary depending on the presence of modifier genes that may differ between embryos on a mixed 129:Bl6 genetic background.

Rb mosaic retinas have reduced photoreceptor layer

Use of the paternally-inherited *NesCre1* transgene led to complete deletion of *Rb* in the retina, but it also causes perinatal lethality (MacPherson et al. 2003). However, due to imprinting effects, a lower degree of Cre-mediated excision occurs with maternal *NesCre1* inheritance (Fan et al. 2001). Reporter analyses revealed that maternal *NesCre1* inheritance leads to a mosaic expression of Cre through all layers of the retina that was highly variable from animal to animal (Supplemental Figure 1 b,c). Use of maternal inheritance of the *NesCre1* transgene allowed us to produce viable offspring with a mosaic pattern of *Rb* deletion. These animals were produced at below Mendelian frequency (approximately 41% of the expected frequency); we refer to them below as "*NesCre1 Rb* mosaics". Histological analyses of adult *NesCre1 Rb* mosaic retinas revealed a dramatic reduction in the number of cells in the photoreceptor-containing outer nuclear layer (Fig 2c,d). The outer segments of the photoreceptors also appeared diminished. The thickness of the *NesCre1 Rb* mosaic outer nuclear layer was half the thickness of controls (Figure 2c,d). This phenotype was similar in *Rb* mosaic mice that were 3 weeks old and those near 4 months old, suggesting that most photoreceptor loss occurred in the first weeks of life. When *Rb* mosaics were examined at postnatal day 3, a time of high levels of photoreceptor generation, we observed high levels of apoptosis (not shown, see similar data in Fig. 5). In addition to the loss of photoreceptors in adults, we found subtle defects in the inner nuclear cells in the *Rb* mosaics. We observed some disorganization in the inner nuclear layer, with clusters of ectopic inner nuclear layer cells in the outer plexiform layer approaching the photoreceptors (Fig. 2c).

Rb mosaics were aged to determine if animals would develop retinal tumors or early lesions. *Rb* mosaics had an average lifespan of 107 days (S.D.= +/-19 days, n=12), and in all cases became moribund due to pituitary tumor development. No retinoblastomas were detected in *Rb* mosaics adults, and we did not observe histological evidence of early retinal tumors. To determine if loss of *Rb* could cooperate with *p53* mutation in apoptosis suppression and tumor development, we bred *NesCre1 Rb* mosaics onto a *p53*-/- background. The photoreceptor layer was still depleted in the absence of *p53* (Fig. 2c,d), and, consistent with the findings that *p53* is not mutated in human retinoblastomas, retinal tumors did not occur in *p53*-/-; *NesCre1 Rb* mosaic compound mutant animals (n=5). However, focal dysplasia was found in some retinas, reminiscent of the retinal dysplasia observed previously in *p53*-/-*Rb*+/- animals (Williams et al. 1994a). *p53*-/-; *NesCre1 Rb* mosaic animals were sacrificed due to tumor burden from pituitary and other non-retinal tumors at an average lifespan of 92+/-15 days. Thus, we conclude that *p53* mutation does not suppress apoptosis or cooperate with *Rb* loss in retinoblastoma formation in the mouse.

Increased severity of retinal phenotypes upon compound mutation of *Rb* family members

We were interested in examining the possible role of the *Rb* family members *p107* and *p130* in compensating for *Rb* deletion in retinal progenitors. Therefore, we bred *NesCre1(p) Rb* mutants onto a *p107* or *p130*-deficient background to generate embryos with retinas completely lacking *Rb* and either *p107* or *p130*. We observed massive retinal dysplasia late in embryonic development (E18.5) in the absence of both *Rb* and

p107 (Fig. 3a). Active Caspase3 immunostaining revealed extensive apoptosis throughout the compound *Rb/p107* mutant retinas (Supplemental Fig. 2a) This phenotype of retinal dysplasia and high levels of apoptosis is similar to the embryonic retinal phenotype in *Rb-/-;p107-/-* chimeras (Robanus-Maandag et al. 1998). Interestingly, we did not observe extensive retinal disorganization at this time in development in embryos lacking both retinal *Rb* and *p130* (Fig. 3a) and the levels of apoptosis in *Rb/p130* mutant retinas were similar to levels with *Rb* mutation alone (Supplemental Fig. 2b). Homozygous mutation of either *p107* or *p130* alone did not lead to retinal phenotypes (not shown).

The *NesCre1(p) Rb* deletion strategy also allowed us determine whether compensatory upregulation of *Rb* family members occurs in *Rb*-deficient embryonic retinas. Extracts of microdissected embryonic retinas from *Rb* mutant, *Rb/p107*, and *Rb/p130* compound mutants and controls were subjected to Western blot analysis. Mutation of p107 or p130 alone did not lead to alterations in the levels of pRB (Fig. 3b) and in contrast to earlier data from other systems (Hurford et al. 1997), p107 was not substantially upregulated at the protein level in response to *Rb* loss. We did find a slight increase in p130 levels with combined *Rb* and *p107* deletion, but not with *Rb*-deletion alone (Fig. 3c). Interestingly, in *Rb*-deficient retinas there was a partial mobility shift to a faster migrating form of p107 compared to controls (Fig. 3c). An even greater p107 mobility shift to the faster migrating form was found in retinas lacking both *Rb* and *p130* (Fig. 3c). To determine if the altered mobility of p107 with *Rb* and *p130* mutation corresponded to a change in p107 phosphorylation, we treated control retina samples with phosphatases. Phosphatase treatment resulted in a complete shift from the slower migrating (hyperphosphorylated) form to the faster migrating (hypophosphorylated) form

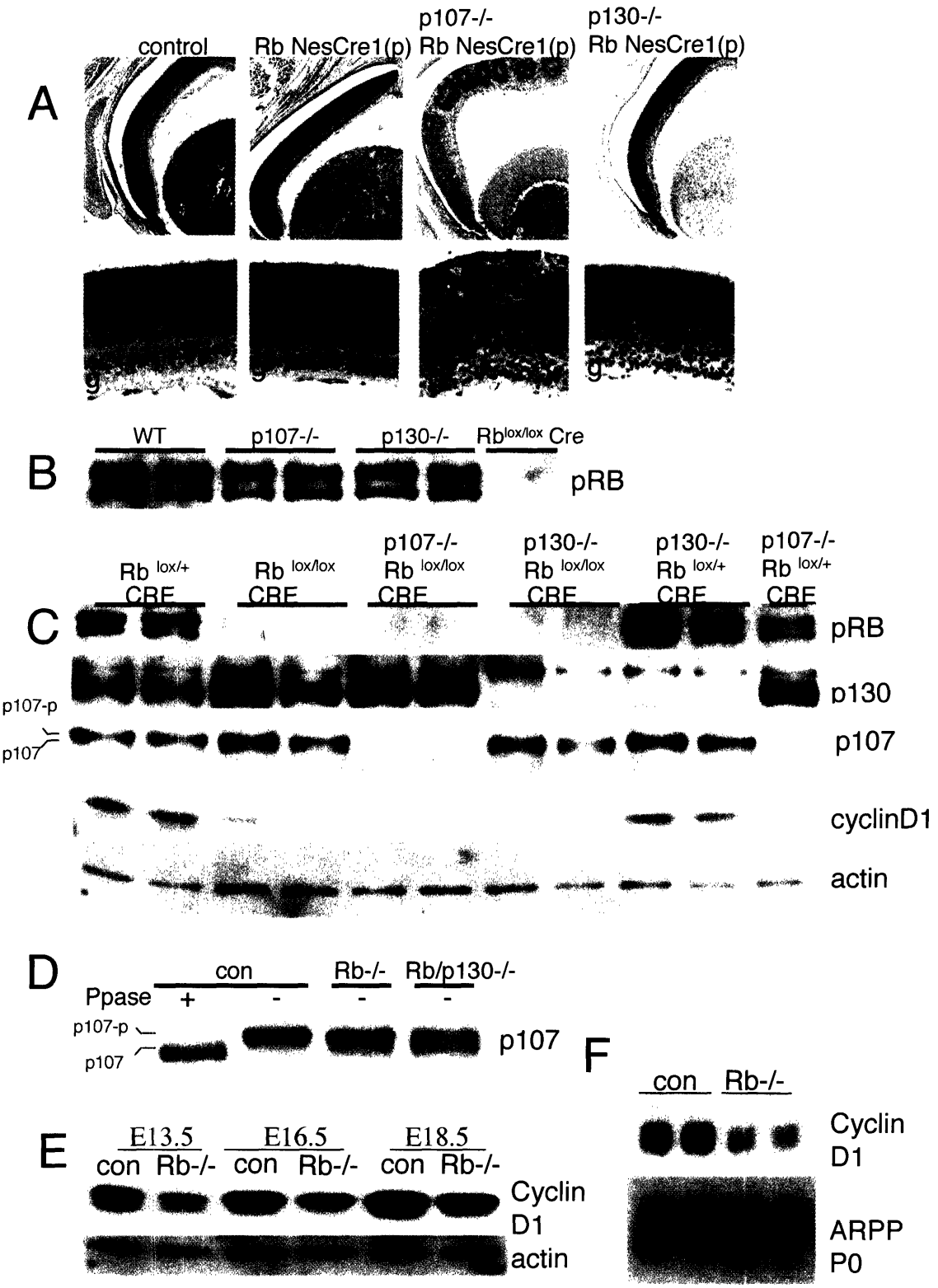
A
control
Rb NesCre1(p)
p107-/-
Rb NesCre1(p)
p130-/-
Rb NesCre1(p)
g
g
g
B
WT
p107-/-
p130-/-
Rblox/lox Cre
pRB
C
Rb lox/+ CRE
Rb lox/lox CRE
p107-/- Rb lox/lox CRE
p130-/- Rb lox/lox CRE
p130-/- Rb lox/+ CRE
p107-/- Rb lox/+ CRE
pRB
p130
p107-p
p107
p107
cyclinD1
actin
D
con
Rb-/-
Rb/p130-/-
Ppase
+
-
-
-
p107-p
p107
p107
E
E13.5
E16.5
E18.5
con
Rb-/-
con
Rb-/-
con
Rb-/-
Cyclin D1
actin
F
con
Rb-/-
Cyclin D1
ARPP P0

Figure 3. Expression of retinoblastoma family members and cyclin D1 in response to *Rb* loss. A) Hematoxylin and Eosin staining reveals *p107* deficiency leads to massive dysplasia in *NesCre1(p) Rb* mutant E18.5 retinas B) pRB expression in wild-tye, p107-/- or p130-/- E18.5 retinas. C) Biochemistry of *NesCre1(p) Rb*-deficient retinas at E18.5. Note p107 shift in mobility and decreased cyclin D1 expression with either *Rb* or *Rb* and *p130* deletion D) Treatment of E18.5 control retinas with calf-intestine- and λ-phosphatases (Ppase) leads to shift from hyperphosphorylated to hypophosphorylated p107 that comigrates with mobility-shifted p107 from *Rb*-deficient or *Rb* and *p130*-deficient E18.5 retina extracts E) Western blot showing cyclin D1 levels decrease in absence of *Rb* in retinas collected at E13.5, E16.5 and E18.5. F) Northern blot showing cyclin D1 downregulation at RNA level in E18.5 *Rb*-deficient retina. Note that for D-F, "con" refers to *Rb lox/+ NesCre1(p)* controls while "Rb-/-" refers to *Rb lox/lox NesCre1(p)* mutants. n, neuroblastic layer; g, ganglion cell layer

of p107 (Fig. 3d). These data suggest that compensation in response to *Rb* loss or *Rb* and *p130* loss may involve regulation of an upstream mediator of p107 phosphorylation and increased p107 activity.

Downregulation of cyclin D1 in the absence of Rb and family members

Cyclin D1 is expressed at extremely high levels in the developing retina in the mouse (Sicinski et al. 1995). Because cyclin D1 can regulate *p107* phosphorylation (Beijersbergen et al. 1995), we examined the levels of cyclin D1 in retinas lacking *Rb*. As shown in Figure 3, levels of cyclin D1 decreased with *Rb* mutation, and further decreased with additional mutation of *p130* (Fig. 3c). *p107* mutation led to cyclin D1 downregulation even with an intact *Rb* allele present (Fig. 3c last lane). Downregulation of cyclin D1 in response to *Rb* loss occurred at each time point in retinal development examined between E13.5 and E18.5 (Fig. 3e). Northern blot analysis on embryonic retinal RNA revealed that the decrease in cyclin D1 expression in *Rb*-mutant tissue occurred at the level of RNA (Fig. 3f). This decrease in cyclin D1 levels correlated with p107 hypophosphorylation in the absence of pRB or pRB and p130. Thus, we hypothesize that a feedback mechanism involving *cyclin D1* may act to dampen the effects of *Rb* mutation. This mechanism could help prevent retinoblastoma development following *Rb* mutation in the mouse.

Bilateral retinoblastomas in *p130-/- NesCre1* Rb mosaics

In order to further pursue the role of *p107* and/or *p130* in compensating for Rb loss, we attempted to generate *NesCre1 Rb* mosaics lacking *p107* or *p130*. However, through

breeding strategies from which 22 compound mutant *p107-/- NesCre1 Rb* mosaics would be expected based on Mendelian ratios, no adult double mutant mosaics were produced. Thus, we conclude that *p107-/- NesCre1 Rb* mosaics die prior to weaning age. In contrast, we were able to generate viable *NesCre1 Rb* mosaics on a *p130-/-* background. Five viable *p130-/- NesCre1 Rb* mosaics (out of an expected Mendelian frequency of 11 animals) were generated and followed for tumor development. Strikingly, all 5 of the double mutant animals developed retinoblastomas, 4 of these being bilateral. Mice were sacrificed when body condition was poor, or if gross retinal tumor burden was apparent; the average tumor-free lifespan in these animals was 79 days (S.D.= +/- 38 days).

Histological analyses of retinoblastomas revealed very similar histology to the human tumor (Figure 4a-f). The tumors were sometimes aggressive, invading surrounding muscle (Fig. 4b). All of the retinoblastomas examined had rosettes similar to Homer-Wright rosettes present in human retinoblastomas (Fig. 4d,f) and had foci with high levels of apoptosis and mitoses. The mouse tumors were always found in the inner nuclear layer, usually filling the area behind the lens. One case showed a mixed pattern of growth as tumor was also found between the photoreceptor outer segments and retinal pigment epithelium (not shown).

Immunostaining with different retinal cell markers showed positive staining for the amacrine marker syntaxin (Fig. 4g). This is in agreement with results found in chimeras with mutation of *p107* and *Rb* in which tumors were also syntaxin positive (Robanus-Mandaag et al, 1998). To explore the possibility that these tumors were of the amacrine lineage further, we immunostained them for calretinin, a marker of AII amacrine cells. As shown in figure 4, the mouse retinoblastomas were intensely

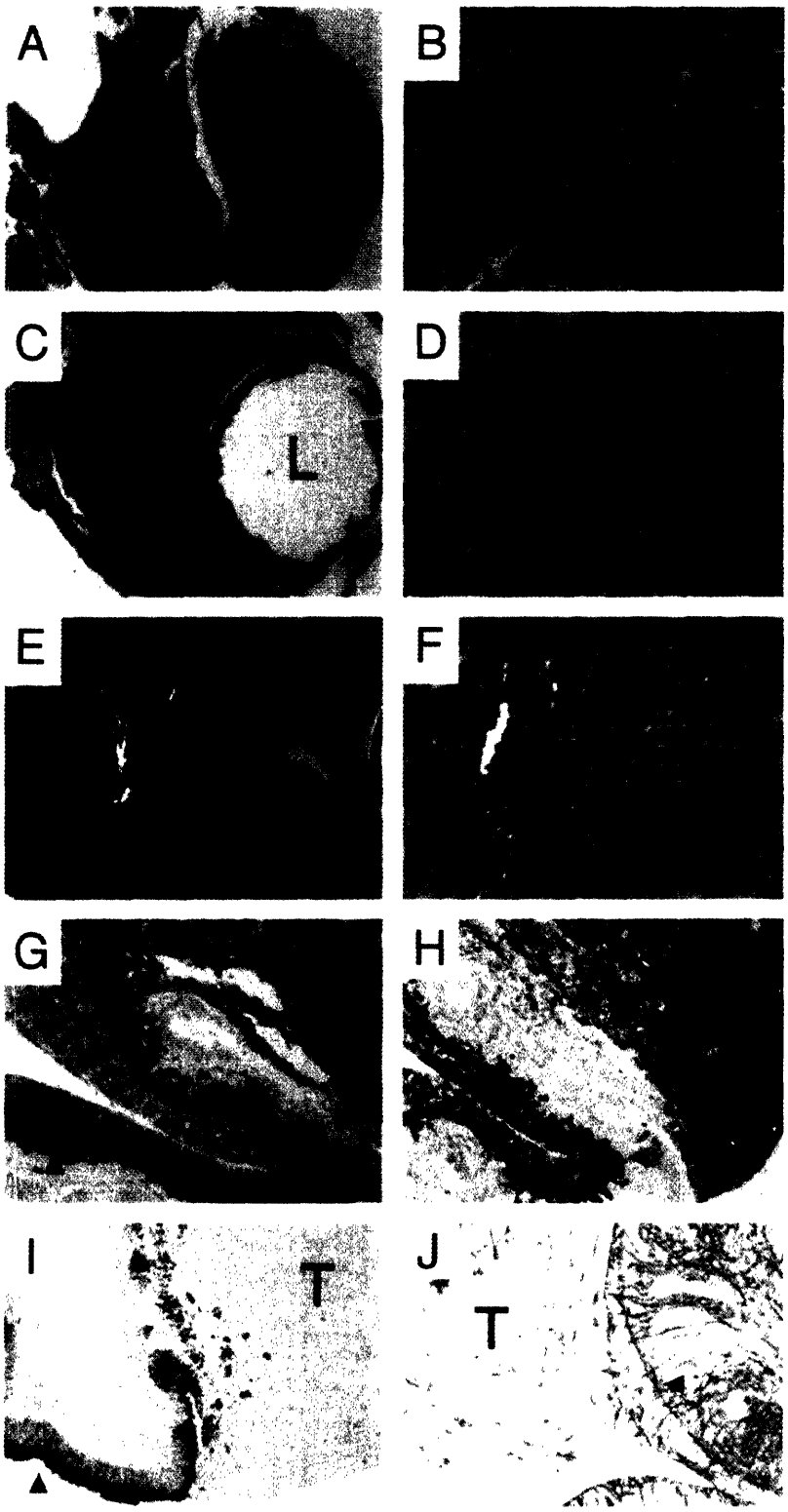
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Figure 4. Retinoblastomas in *p130-/-; NesCre1 Rb* mosaics. A-F) Hematoxylin and eosin staining of three retinoblastomas A,B) invasive retinoblastoma at 40X (A) and 400x (B) showing tumor invasion into muscle. C,D) Retinoblastoma at 40X (C) with rosette like structures visible at 400x (D). E,F) Retinoblastoma (100x) with rosette like structures visible at 400x (F). G) Immunohistochemistry of retinoblastoma from E) with amacrine marker syntaxin showing positive staining throughout tumor (T) (100X). Arrowhead shows area of normal staining of the amacrine cell bodies and processes in the retina inner nuclear layer. H) Calretinin immunohistochemistry labeling a subset of amacrine cells in the normal area of retina (arrowhead), with strong staining in tumor (T). I) IRBP immunohistochemistry labeling photoreceptor outer segments (arrowhead) but not staining the tumor (T). J) GFAP immunohistochemistry labeling Muller Glia, with a pattern indicative of gliosis in the mutant retina (arrowhead) with some cells in the area of the tumor staining positively. The lens is indicated (L).

calretinin positive (Fig. 4h). The tumors did not stain for interphotoreceptor retinoid-binding protein (IRBP), expressed in photoreceptor outer segments (Fig. 4I), the bipolar cell marker protein kinase C-α (PKC-α), or the retinal ganglion cell marker Brn3b (not shown). We also performed immunostaining for glial fibrillary acidic protein (GFAP), which is upregulated in Muller glia exhibiting gliosis, and found some GFAP-positive cells, both in adjacent, normal retina and, infrequently, in the tumor (Fig. 4j). This staining pattern is suggestive of reactive gliosis, as the vast majority of cells in the area of the tumors were GFAP negative. Our data and the results of Robanus-Mandaag et al (1998) indicate that mutation of either *p107* or *p130* on a *Rb*-deficient background can lead to retinoblastomas of similar histology and, likely, histogenesis.

Apoptosis after retinal-specific *Rb* deletion

In order to study the specific effects of *Rb* deletion in the major retinal cell types, including amacrine cells, we crossed the $Rb^{lox/lox}$ mice to *alpha-Cre* transgenic mice. In this strain *Cre* expression is controlled by elements of the *Pax6* promoter, allowing for retinal-specific gene deletion in retinal progenitors in distal retina as early as E10.5 (Marquardt et al. 2001). Complete gene deletion has been reported in the distal retina, but not in proximal retina (Marquardt et al. 2001). This approach allowed us to characterize the specific developmental defects in retinal cell types that might have been less apparent or more variable in the *Rb* mosaics.

As shown in Figure 5, retinas from three week-old *alpha-Cre* $Rb^{lox/lox}$ animals exhibited widespread photoreceptor loss. To determine if apoptosis contributed to the photoreceptor phenotype, we performed immunostaining for active caspase3 at various

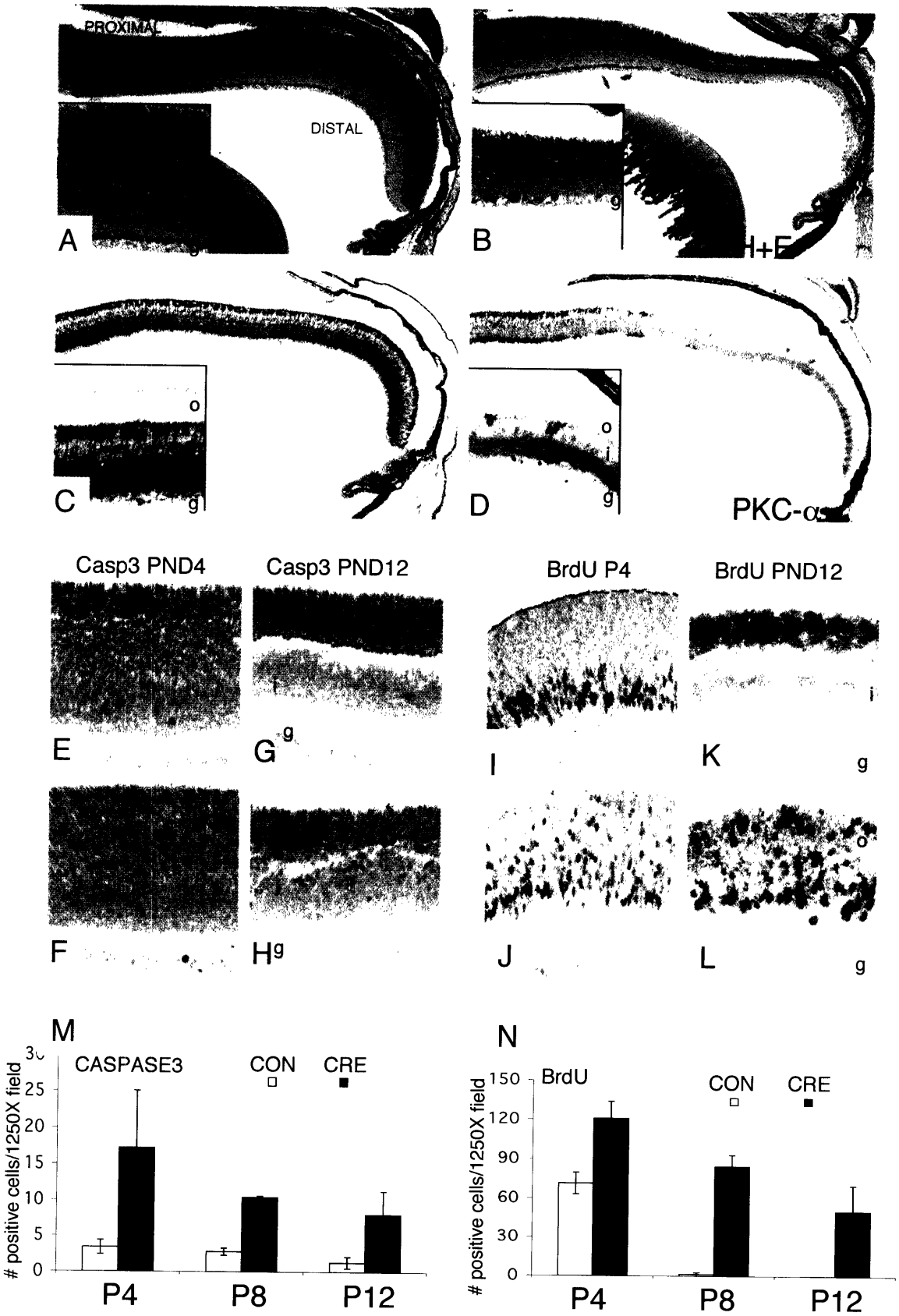

PROXIMAL
DISTAL
A
B
H+E
C
D
PKC-α
Casp3 PND4
Casp3 PND12
BrdU P4
BrdU PND12
E
G
I
K
F
H
J
L
M
CASPASE3
CON
CRE
positive cells/1250X field
P4
P8
P12
N
BrdU
CON
CRE
positive cells/1250X field
P4
P8
P12

Figure 5. Proliferation, apoptosis and cell loss in *Rb*^{loxlox} *alpha-Cre* distal retina. A) Hematoxylin and eosin staining of 3-week control and B)*Rb*^{loxlox} *alpha-Cre* retinas showing reduced photoreceptor layer and inner nuclear layer (100X). Proximal and distal retina is labeled. Cre expressed in the distal retina deletes *Rb* in B). Inset: Distal retina (400x). C) Immunostaining with anti-PKCα, labeling rod bipolar cells in control and D) *Rb*^{loxlox} *alpha-Cre* 3 week retina (100X). Inset: Distal retina (400x). E-F) Active caspase3 immunostaining at PND4 in control (E) or *Rb*^{loxlox} *alpha-Cre* (F) distal retina. G-H) Active Caspase3 immunostaining at PND12 in control (G) or *Rb*^{loxlox} *alpha-Cre* (H) distal retina. I-J) BrdU analysis at PND4 in control (I) or *Rb*^{loxlox} *alpha-Cre* (J) distal retina. K-L) BrdU analysis at PND12 in control (K) or *Rb*^{loxlox} *alpha-Cre* (L) distal retina. Note the complete absence of BrdU incorporation in controls, with many positive cells in *Rb*^{loxlox} *alpha-Cre* mutants at this time. Quantification of Caspase 3 immunostaining (M) and BrdU analysis (N). BrdU was injected 1 hour prior to animal sacrifice.

g, ganglion cell layer; i, inner nuclear layer; o, outer nuclear layer.

times during development. While few apoptotic cells were apparent in controls (Fig. 5e), extensive apoptosis was evident throughout the *Rb*-deficient distal retina at postnatal day 4 (PND4) (Fig. 5f,m). At PND12, the photoreceptor-containing outer nuclear layer was visible in control animals; cells with photoreceptor-like morphology were present and no significant apoptosis in the outer nuclear layer was detected (Fig. 5g,m). However, in the *alpha-Cre Rb* $^{lox/lox}$ distal retina, pyknotic nuclei (not shown) and caspase 3 immunopositive cells were observed in the outer nuclear layer (Fig. 5h). This suggests that photoreceptors are born, but die at some point in differentiation in the absence of *Rb*. Apoptosis was also found in the inner part of the *Rb* mutant retina at PND4 and PND12 (Fig. 5f,h).

Given the observed cell cycle defects in *NesCre1(p)* $Rb^{lox/lox}$ retinas at E18.5 (Fig. 1,2), we examined cell cycle activity in post-natal embryos. DNA synthesis in the normal murine retina has been reported to be completed by PND11 (Young 1985), and we did not observe BrdU positive cells in control retinas at PND12 (Fig. 5k). In contrast, in *Rb* mutants very high levels BrdU positive cells were present at this stage, indicative of inappropriate S-phase entry (Fig. 5l,m). We noted that the inappropriate BrdU incorporation and apoptosis was not restricted to the photoreceptor-containing outer nuclear layer, but was found throughout the retina (Fig. 5j). An overall increase in BrdU staining was found between PND4 and 12 in Rb mutants (Fig. 5j,l,m).

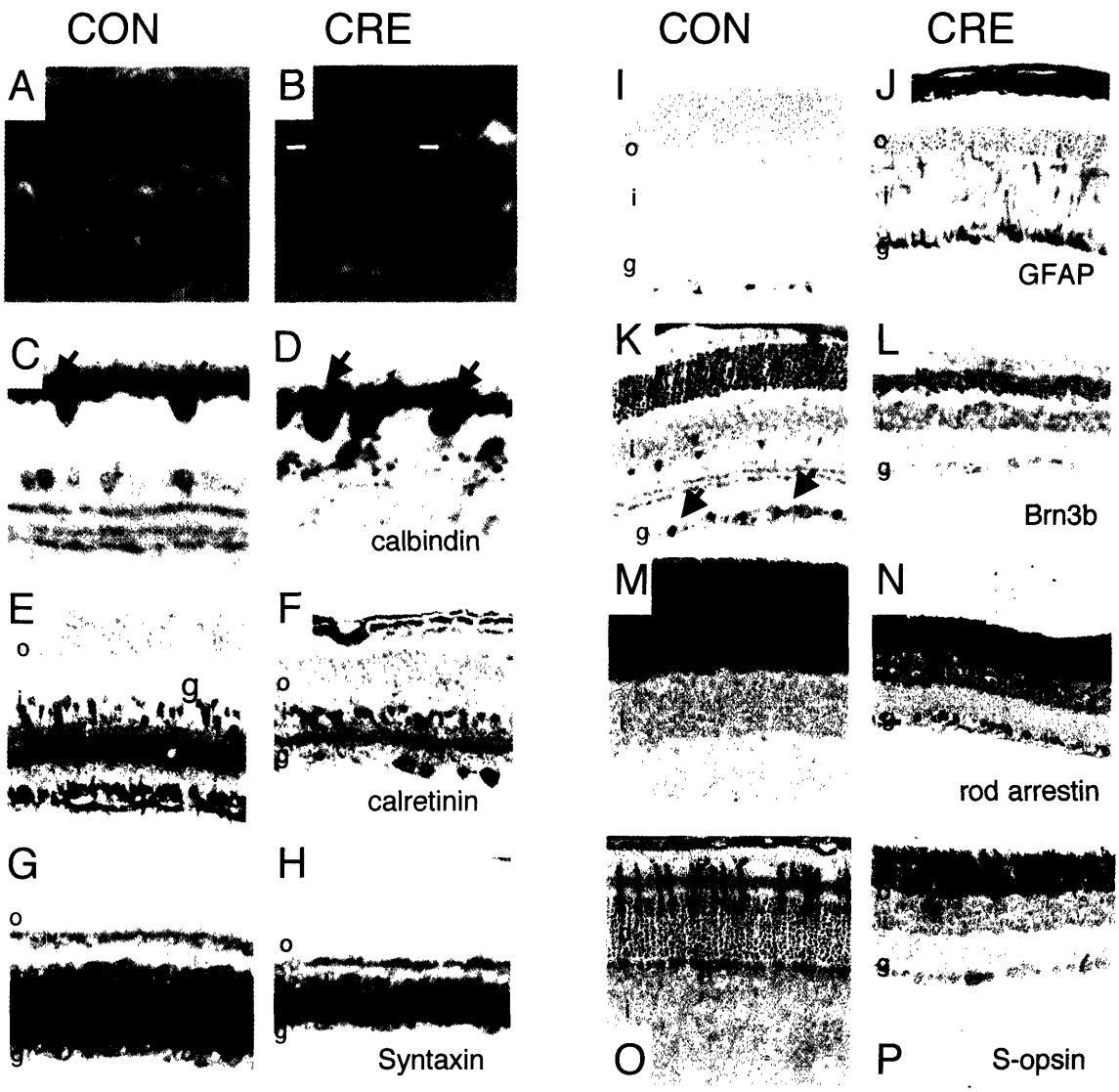
Rod bipolar loss and inner nuclear layer defects with *Rb* deletion

In addition to the reduced outer nuclear layer (ONL), the inner nuclear layer (INL) of *alpha-Cre Rb* mutants was clearly affected although to a lesser extent than the ONL (Fig

5a, b). Along with the diminished INL, the corresponding inner plexiform layer of axons was also reduced. We used immunohistochemical analyses with cell type-specific markers to further characterize effects of *Rb* deletion on development of specific cell types at 3 weeks of age when retinal histogenesis is complete. Labeling with PKC-α, a rod bipolar cell marker (Fig. 5c, d), revealed that *Rb* deletion led to near complete loss of rod bipolar neurons (Fig. 5d). Loss of rod bipolar cells occurred prior to PND12, as PKC-α positive cells were also absent at this time (not shown).

In contrast to the stark depletion of many photoreceptors and rod bipolar cells with *Rb* gene mutation, some INL cell types survived the absence of *Rb*. In the 3-week old *alphaCre Rb* mutants, we observed aberrant, very large cells in the upper part of the INL that were never seen in controls (Fig. 6a, b). Calbindin immunostaining was consistent with these cells being in the horizontal cell lineage (Fig. 6c, d).

In control-mice, calbindin also labels a subset of amacrine cells in the INL and displaced amacrine cells and ganglion cells in the GCL (Fig. 6c). The post-natal *alphaCre Rb* mutants had calbindin-positive amacrine cells as well, but there was clear disorganization and loss of laminar structure in the inner plexiform layer (Fig. 6d). Calretinin immunostaining of AII amacrine cells also indicated a loss of laminar structure in the inner plexiform layer in *alphaCre Rb* mutants (Fig. 6e, f), syntaxin staining revealed a slight decrease in the thickness of the amacrine layer compared to controls (Fig. 6g,h). Given the presence of amacrine cell markers in murine retinoblastomas (see Fig. 4), the survival of many Rb-deficient amacrine cells may be highly significant.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rod arrestin
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Syntaxin
S-opsin

Figure 6. Immunohistochemical characterization of retinal cell types upon *Rb* deletion. A) Hematoxylin and Eosin staining on inner nuclear layer (1000X) of distal retina from controls and B) Rb^{loxlox} *alpha-Cre* mutants at three weeks of age. Note the decreased INL thickness in the mutant INL and the presence of cells with abnormally large nuclei (arrows) in mutant. C) Calbindin immunostaining of distal retina from controls and D) Rb^{loxlox} *alpha-Cre* mutants labeling horizontal cells strongly in the upper INL as well as some lower amacrine cells. E) Calretinin immunostaining labeling a subset of amacrine cells in controls and F) Rb^{loxlox} *alpha-Cre* mutants. G) Syntaxin immunostaining labeling amacrine cells and processes in controls and H) Rb^{loxlox} *alpha-Cre* mutants. I) GFAP immunostaining of distal retina in controls or J) Rb^{loxlox} *alpha-Cre* mutants. Note the presence of GFAP positive Muller glia vertical processes only in the Rb^{loxlox} *alpha-Cre* mutants (J). In controls, GFAP signal is restricted to astrocytes in the inner limiting membrane. K) Brn3b immunostaining labeling retinal ganglion cells in the GCL in controls (arrows) and L) very rarely in Rb^{loxlox} *alpha-Cre* mutants. M) Rod arrestin (S-Ag) staining in controls and N) Rb^{loxlox} *alpha-Cre* mutants. O) S-opsin signal in cone subset in controls and P) Rb^{loxlox} *alpha-Cre* mutants.

g, ganglion cell layer;i, inner nuclear layer; o, outer nuclear layer.

GFAP is upregulated in Muller glia in response to retinal injury, and most mouse models of photoreceptor degeneration also show GFAP upregulation. Staining with GFAP showed that Muller glia were also present and were in an active state indicative of gliosis (Fig. 6i,j) in the *alphaCre Rb* mutants at 3 weeks of age. Consistent with the observed apoptosis in the Rb-deficient retinal ganglion cell layer at E18.5, we found that *alphaCre Rb* mutants had only rare cells positive for Brn3b, a marker of retinal ganglion cells (Fig. 6k,l). Finally we examined photoreceptor marker expression to determine if the photoreceptor loss affected rods and cones. Some rod photoreceptors survived Rb loss, staining positively for rod arrestin in the absence of Rb, though the thichness of this layer was dramatically reduced (Fig.6 m,n). Decreased photoreceptor layer thickness is likely due to rod loss, as we did not observe a similar loss of S-opsin positive cones in the absence of Rb (Fig. 6o,p) and the vast majority of photoreceptors in the murine retina are rods.

In summary, *Rb* deletion in the retina leads to strong effects on retinal ganglion cells, rod photoreceptors and bipolar cells. Horizontal cells, Muller glia and amacrine cells can survive in the absence of *Rb* function, although disorganization in the inner nuclear layer is apparent and horizontal cells show cytologic abnormalities. We conclude that *Rb* is a critical regulator of cell cycle exit in the retina and that the consequences of failure to exit the cell cycle are cell type specific, with apoptosis a frequent endpoint for some cells. The survival of some *Rb*-deficient amacrine cells may help explain the development of retinoblastomas with amacrine cell features in mice mutant for *Rb* and *p130* (Fig. 4g,h).

Discussion

In this report we describe a critical role for the retinoblastoma gene in normal retinal development. *Rb* deletion in this tissue leads to failure in cell cycle exit and p53-independent apoptosis in multiple cell types. Importantly, *Rb* mutation combined with *p107* and with *p130* mutation caused retinal dysplasia or retinoblastomas with amacrine cell characteristics. While most major cell types were affected by *Rb* mutation, amacrine cells were able to develop and survive.

We and others have previously described apoptosis in response to Rb deletion during development of the CNS (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Wu et al. 2003). However, apoptosis in the Rb germline mutant CNS is secondary to hypoxia caused by abnormal placental development (MacPherson et al. 2003; Wu et al. 2003). In contrast, we have demonstrated that retinal cell apoptosis in the absence of *Rb* is *p53*-independent and a direct consequence of *Rb* deletion. Previous studies involving expression of human papillomavirus E7 (which binds and inactivates all pRB family members) in the photoreceptor compartment also showed links between loss of Rb family function and apoptosis (Howes et al. 1994). Interestingly, retinal E7 expression on a p53-mutant background led to retinal tumors in the photoreceptor layer (Howes et al. 1994) and photoreceptor expression of SV40 T antigen (which inhibits the pRB family as well as p53) also caused photoreceptor layer tumors (al-Ubaidi et al. 1992). However, we have shown that combined mutation of *Rb* and *p53* does not lead to retinoblastoma development, a result which is consistent with the fact that *p53* has not been found to be mutated in human retinoblastomas (Gallie et al. 1999).

While mutation of *p53* did not strongly cooperate with *Rb* mutation in *NesCre1* *Rb* mosaics, we did observe striking cooperativity between *Rb* and *p130* in retinal tumor development. All five *p130-/- NesCre1* *Rb* mosaic animals examined developed retinoblastomas, which had remarkable histological similarities to their human counterparts. Bilateral tumors were present in 4 of 5 of these animals. Beyond the similarities in tumor histology, the mouse tumors were also notable due to the presence of Homer-Wright rosettes, high apoptotic and mitotic levels, and tumor invasiveness. However, the mouse retinoblastomas did not contain Flexner-Wintersteiner rosettes, which have been found in some human retinoblastomas and have evidence of photoreceptor differentiation. All of the tumors in our model appeared to have inner nuclear layer characteristics, although one tumor also had outer layer growth between the photoreceptor outer segments and pigment epithelium. Of note, Bellan et al (2001) have reported that lack of p130 expression was strongly associated with poor differentiation in human retinoblastomas. Thus, our model may recapitulate this subset of human retinoblastomas. A thorough mutational analysis of p130 in human retinoblastomas has not been performed. However, it is interesting to note that the p130 genomic region (at 16q12), is frequently lost in human retinoblastomas (Mairal et al. 2000; Chen et al. 2001; Herzog et al. 2001; Lillington et al. 2003).

These data complement those of Robanus-Mandaag et al (1998) who showed that 5/7 chimeras with *Rb/p107-/-* cells developed retinoblastomas. We could not generate *p107-/-;NesCre1* *Rb* mosaic adult mice due to early lethality. However, it is very likely that retinoblastomas would have developed had these animals survived given the extensive retinal dysplasia in embryonic retinas lacking *Rb* and *p107*. Also, Chen et al

(2004) find that retinal-specific conditional mutants lacking *Rb* and *p107* develop retinoblastomas at high frequency. The characteristics of the *p130-/- NesCre1 Rb* tumors reported here and those from *Rb/p107* mutant retinas (Robanus-Maandag et al. 1998, Chen et al, 2004) are similar, and these studies point to an amacrine cell (or precursor) as a cell of origin in retinoblastoma. Because the necessary animals can be generated by breeding, our retinoblastoma model using maternal expression of the *NesCre1* transgene on a *p130-/-* background is more tractable than chimera-based models and should facilitate further analysis of the genetics and cell biology of retinoblastoma development.

A simple explanation for the apparent functional compensation between *Rb* family members in the developing retina is that in response to *Rb* loss p107 and/or p130 is upregulated to provide additional cell cycle inhibitory function. In fact in *Rb*-deficient embryo fibroblasts, muscle progenitors and other cell types, p107 levels are clearly elevated (Schneider et al. 1994; Hurford et al. 1997). In whole tissue extracts from *Rb*-deficient retinas, however, we found that the absolute levels of p107 were not increased. Instead, there was a subtle shift in the phosphorylation state of p107 upon *Rb* deletion to more of the active, hypophosphorylated form. Cyclin D1 is expressed at extremely high levels in the developing retina and the hypoplastic retinas in mice lacking *cyclin D1* points to a critical role for *cyclin D1* in retinal cell proliferation (Fantl et al. 1995; Sicinski et al. 1995). Interestingly, we observed that the expression of cyclin D1 was reduced in *Rb*-/-retinas, which could account for p107 hypophosphorylation. Both the p107 phosphorylation shift and decrease in cyclin D1 levels were magnified with mutation of both *Rb* and *p130*. We postulate that cyclin D1 is downregulated in order to induce the activity of other *Rb* family members. This mechanism could suppress

tumorigenic effects of *Rb* loss alone and provide a selective force for mutation of *p107* and *p130*. This is the first demonstration of the negative control of *cyclin D1* expression by *Rb* in vivo and extends observations made by others in *Rb*-deficient cells in vitro (Muller et al. 1994; Takebayashi et al. 2003). An important question is the nature of the *Rb* activity for which *p107* might compensate that is essential for suppression of retinoblastoma. We showed that *Rb* is important for cell cycle exit in the retina with *Rb* loss leading to ectopic S-phase entry and extensive S-phase entry when normal cell division had stopped. Interestingly, in a study of the effects of retinal-specific deletion of both *Rb* and *p107*, there was an exacerbation of cell cycle exit defects compared to *Rb* mutation alone, although *Rb/p107-/-* cells did eventually exit the cell cycle (Chen et al, 2004). It is possible that in the murine retina lacking *Rb* alone, active hypophosphorylated p107 may allow the retinoblastoma precursor cell to exit the cell cycle before other events critical for retinoblastoma development occur.

A striking phenotype apparent in *Rb* mosaics and in *alpha Cre* conditional *Rb* mutants was a dramatic reduction in the photoreceptor cell layer. *Rb*-deficient photoreceptors were born but apoptosis could be found in the differentiating photoreceptor layer at P12, and rod photoreceptor loss was found in adults. This was surprising given a report by Vooijs et al. (2002), who used the photoreceptor-specific IRBP-promoter to drive *Cre* mediated *Rb* deletion in the retina and did not report phenotypic effects. In that model *Cre* was expressed in only a fraction of photoreceptors, and, thus, may have led to only a partial decrease in the photoreceptor layer. Alternatively, the specific timing of deletion could be critical. In our studies *Cre* was expressed in early retinal progenitors starting near E10.5 (Marquardt et al. 2001), while

IRBP-driven CRE expression was first detected at E14.5. (Vooijs et al. 2002). Recently, Zhang et al (2004) have reported that *Rb* is critical for proliferation and for rod photoreceptor development in the mouse. This group used an in vitro retinal explant system to study *Rb* retinal function, and they also deleted *Rb* in the retina using retinal-specific Cre expression. However, Cre-mediated *Rb* deletion occurred in patches and only defects in proliferation and in rod photoreceptor development were reported (Zhang et al. 2004). Also, this group did not find increased apoptosis in their system, which contrasts with the results of our study, and those of the Bremner lab who have also generated retinal-specific *Rb*-mutant mice (Chen et al, 2004). Here, we have been able to achieve more complete *Rb* deletion, in vivo, allowing the discovery of much broader cell-type specific effects of *Rb* deletion in the retina and a critical role for *Rb* in suppression of retinal apoptosis.

We characterized the effects of *Rb* deletion on the major cell types of the mouse retina. In addition to loss of rod photoreceptors, we found high levels of apoptosis in the retinal ganglion cell layer at a time when differentiation is occurring. Strikingly, we also found that rod bipolar cells were rarely present in *Rb* mutant adult retinas. This phenotype correlated with the high levels of apoptosis in *Rb* mutant retinas at various times in development. However, in contrast to the photoreceptor compartment where apoptotic cells were clearly evident at PND12, we have not determined the time at which the bipolar cells are lost. It remains possible that *Rb* deletion could affect the birth of these cells or cause apoptosis very early on in bipolar cell differentiation. Our data show that *Rb* deletion causes a failure in cell cycle exit and cell death, either as an indirect

consequence of these cell cycle defects or more direct functions of *Rb* in regulation of apoptosis.

In the midst of high levels of cell death some *Rb*-/- retinal cells survive. For example, horizontal cells can develop but these often have abnormally large nuclei. This phenotype is reminiscent of Purkinje cells from *Rb*-/-:+/+ chimeras and *NesCre1 Rb* mosaics ((Williams et al. 1994b), D.M and T.J. unpublished) and *Rb*-/- skeletal muscle (Zacksenhaus et al. 1996; de Bruin et al. 2003; MacPherson et al. 2003). In *Rb*-/- skeletal muscle, ectopic S-phase entry occurs in differentiating cells and loss of *Rb* has been linked to endoreduplication cycles and polyploidy (Zacksenhaus et al. 1996). We also found Muller glia to be present and in an activated state associated with upregulation of GFAP. Interestingly, inactivating mutation in *p27* (an upstream regulator of pRB) led to constitutive Muller glia activation with GFAP upregulation (Dyer and Cepko 2000). However, retinal defects involving photoreceptor degeneration usually causes Muller glia activation with GFAP upregulation, and our experiments have not addressed whether the gliosis-like phenotype in the *Rb*-deficient retina is a primary defect or secondary to the photoreceptor loss. We were particularly interested in the fate of the amacrine cells due to the positive staining of the *p130*-/- *NesCre1 Rb* mosaic retinoblastomas for the amacrine cell markers syntaxin and calretinin. Most of the cells remaining in the *alphaCre Rb* mutant inner nuclear layer appeared to be amacrine cells based on syntaxin staining. There appeared to be some decrease in the amount of these cells in *Rb* mutants compared to controls, but clearly many amacrine cells can survive deletion of *Rb*.

The observation of high levels of apoptosis in the developing *Rb*-/- retina is difficult to reconcile with the tumor suppressive function of *Rb* in this tissue. This

observation leads to the possibility that apoptosis would have to be suppressed during tumor initiation or progression, as has been suggested in other settings (Howes et al. 1994; Symonds et al. 1994). Indeed, there is evidence that retinoblastomas do undergo consistent, unknown genetic alterations in addition to *RB* deletion that have been suggested to be linked to apoptosis suppression (Mairal et al. 2000; Chen et al. 2001; Herzog et al. 2001; Lillington et al. 2003). However, our data indicate that mutations in apoptotic pathways may not be necessary for retinoblastoma development. While many cells in the retina, including photoreceptors, rod bipolar cells and retinal ganglion cells respond to *Rb* loss by undergoing apoptosis, amacrine cells and some other cell types can tolerate *Rb* mutation. Thus, the specific tumorigenic effects of *RB* mutation in human retinas and *Rb/p107* or *Rb/p130* in the mouse may be linked to the fate of different cells harboring these mutations. The careful study of this process should finally reveal the origins of retinoblastoma and the exact contribution of loss of *Rb* family function to the development of this tumor.

Materials and Methods

Mice

NesCre1 transgenic mice were obtained from Andreas Trumpp (ISREC, Switzerland) and generation of the *Rb/NesCre1* mutants has been previously described (MacPherson et al. 2003). All embryo studies were performed using paternal inheritance of *Cre*, to achieve complete *Rb* deletion in the retina. *NesCre1 Rb* mosaics are animals in which the *NesCre1* transgene was maternally inherited. *p107, p130, p53* germline mutant mice have been previously described (Jacks et al. 1994; Cobrinik et al. 1996; Lee et al. 1996).

AlphaCre transgenic mice express Cre from elements of the pax6 promoter, as described (Marquardt et al. 2001). Lox-STOP-lox LacZ reporter mice Gt(ROSA)26Sor^{tm1Sor} (Soriano 1999) were obtained from Jackson Laboratories.

Immunohistochemistry, BrdU and TUNEL assays

Eyes were fixed in Bouins solution or 10% neutral buffered formalin (3.7% formaldehyde in PBS) for 24 hours before processing into paraffin. 4uM paraffin sections were cut and processed from xylene through a graded ethanol series to PBS. Unmasking was performed using microwave heating in citrate buffer (0.01M sodium citrate pH 6). Endogenous peroxidases were blocked with 1% H202 and immunohistochemistry was performed with an overnight incubation in the following antibodies: active caspase 3 (Cell Signaling, 1/200); phospho-histone H3 (Upstate, 1/200); calbindin D28 (Chemicon 1/500); syntaxin (Sigma, 1/3000); calretinin (Chemicon, 1/1500); IRBP (polyclonal anti-monkey, gift of Dr. Rachel Caspi, 1/3000); PKCα (BD Transduction Laboratories 1/200); GFAP (DAKO, 1/600); Brn3b (Santa Cruz, 1/100). 5% normal horse serum was used in blocking for all mouse primary antibodies, while 5% normal goat serum was used in blocking for all rabbit primary antibodies. Biotin-conjugated secondary antibodies (Vector Laboratories) were used at a dilution of 1/200 in blocking solution. After secondary antibody binding, detection was performed via a biotin-peroxidase complex (Vectastain ABC, Vector Laboratories) with diaminobenzidine substrate (Vector Laboratories). TUNEL and BrdU were performed as described (MacPherson et al. 2003). For BrdU analysis, an intraperitoneal injection of pups, or for embryo studies, pregnant

mothers, was done with 100μg/g body weight and eyes were collected/fixed one hour later.

ß-Galactosidase Histochemistry

Whole embryos or adult retinas were dissected and fixed in 4% Paraformaldehyde (PFA) for 1hr at 4° C, rinsed and then assayed for β-Galactosidase activity. Tissue was incubated in X-Gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), 5 mmol/L $K_3Fe(CN)_3$, 5 mmol/L $K_4Fe(CN)_6$, 2 mmol/L $MgCl_2$, 0.015% sodium deoxycholate, 0.03% IGEPAL CA-630 in PBS) overnight at 37° C, washed, and postfixed in 10% NBF (3.7% formaldehyde in PBS) at 4° C. Stained tissue was transferred through an ethanol series to xylene, paraffin-embedded and 4μM sections were cut.

Western and Northern blotting

Retinas were microdissected from E13.5 or E18.5 embryos under a Zeiss microscope and immediately frozen on dry ice. For Western blots on E13.5 embryos, four retinas were pooled following embryo genotyping. Protein was isolated by extraction in 100μL lysis buffer (100 mM Tris pH 8, 100 mM NaCl, 1% Nonidet P-40, with Complete protease inhibitor tablets (Roche Molecular Biochemicals), 1mM sodium vanadate, 1mM sodium flouride). For phosphatase treatment, sodium flouride and sodium vanadate were excluded, and 10mM $MgCl_2$ and 1mM dithiothreitol were added to the lysis buffer. Lysates were incubated for 15 minutes with Calf-Intestine Phosphatase and then with λ-phosphatase at 30°C. Western blots were performed as described (MacPherson et al.

2003). Antibodies to the following antigens were used: pRB (BD Biosciences), p107 (C-18 Santa Cruz), p130 (C-20 Santa Cruz), cyclin D1 (Santa Cruz), actin (I-20 Santa Cruz). For Northern blots, pooled E18.5 retinas were lysed in TRIZOL (Invitrogen) using a 30 gauge syringe and RNA was isolated following the manufacturers instruction. 130ng total RNA was loaded, and Northern blotting was performed using standard protocols. cDNAs for cyclin D1 or ARPP P0 were used to generate probes.

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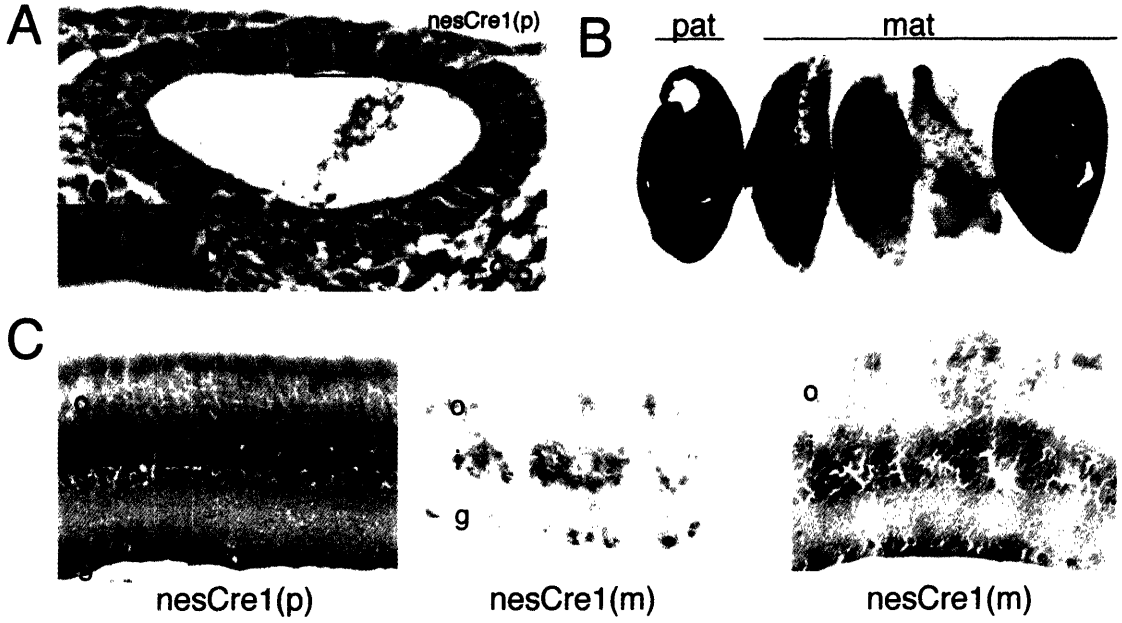
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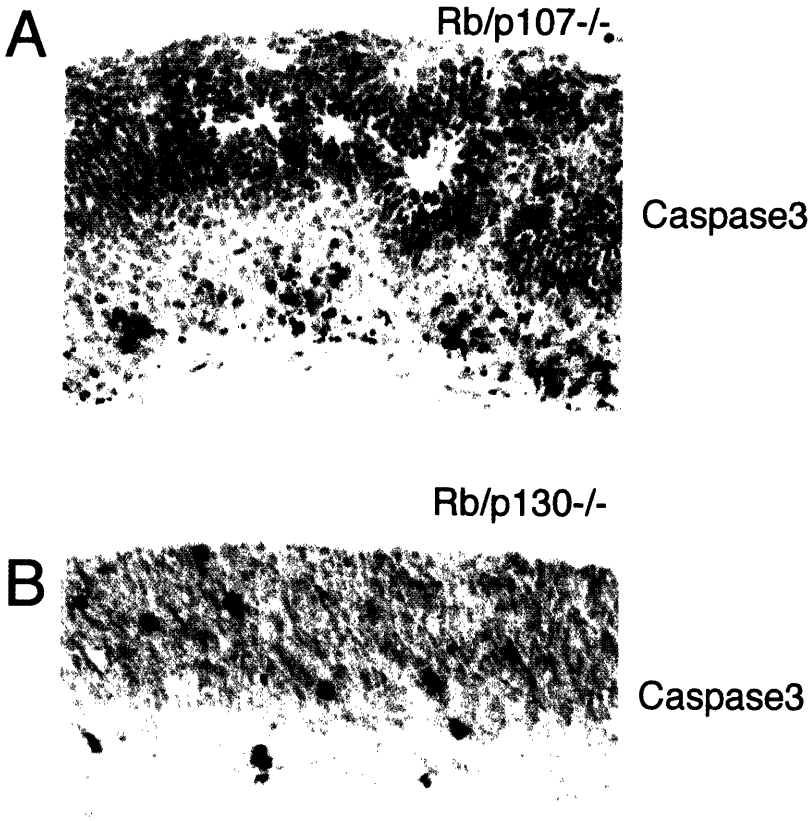
Supplemental Figure 1

A) X-gal staining of E9.5 embryos with paternal inheritance of the *NesCre1* transgene at 400X (with inset at original magnification of 1000x). Embryos were paraffin embedded, sections were cut at 4μM and counterstained with nuclear fast red. B) Whole mount X-gal staining of adult retinas with paternal inheritance (pat) or maternal inheritance (mat) of the *NesCre1* transgene. Retinas stained from four different animals with maternal *NesCre1* inheritance illustrates the extensive variability in reporter gene activity. C) 4μM sections of representative adult retinas from paternally or maternally inherited *NesCre1* that were paraffin-embedded and sectioned following whole mount X-gal staining (400X).


A
Rb/p107-/-
Caspase3
Rb/p130-/-
B
Caspase3

Supplemental Figure 2.

A)Active Caspase-3 immunostaining of compound *p107-/-; $Rb^{lox/lox}$ NesCre1(p)* mutant retinas at E18.5 . *Rb/p107* compound mutant retinas exhibited a mean +/- S.D. of 418+/-102 Caspase3 positive cells per section. (B)Active Caspase-3 immunostaining of compound *p130-/-;$Rb^{lox/lox}$ NesCre1(p)* mutant retinas at E18.5. *Rb/p130* compound mutant retinas exhibited a mean +/- S.D. of 78.1+/-7.6 Caspase3 positive cells per section (similar to the levels shown in Figure 2 with *Rb* mutation alone). n≥3.

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Chapter 4

Defective apoptosis and B-cell lymphomas in mice with p53 point mutation at Ser 23

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The author performed all experiments described in Figures 2 through 7 with technical assistance from Teresa Kim. Jungho Kim generated the Ser23 mutant mice, as described in Figure 1. Roderick Bronson and Mark Fleming provided histopathological expertise in classifying tumors. This work was done in the lab of Tyler Jacks.

Abstract

Phosphorylation of the p53 tumor suppressor at Ser20 (murine Ser23) has been proposed to be critical for disrupting p53 interaction with its negative regulator, MDM2, and allowing p53 stabilization. To determine the importance of Ser23 for the function of p53 in vivo, we generated a mouse in which the endogenous p53 locus was targeted to replace Ser23 with alanine. We show that in MEFs generated from Ser23 mutant mice, Ser23 mutation did not dramatically reduce IR-induced p53 protein stabilization or p53-dependent cell cycle arrest. However, in Ser23 mutant thymocytes and in the developing cerebellum, p53 stabilization following IR was decreased and resistance to apoptosis was observed. Homozygous Ser23 mutant animals had a reduced lifespan but did not develop thymic lymphomas or sarcomas that are characteristic of p53-/- mice. Instead, Ser23 mutant animals died between one and two years with tumors that were most commonly of B-cell lineage. These data support an important role for Ser20/23 phosphorylation in p53 stabilization, apoptosis and tumor suppression.

Introduction

The p53 tumor suppressor becomes stabilized and activated in response to diverse cellular stresses such as DNA damage, hypoxia, ribonucleotide depletion and oncogene activation (reviewed in (Giaccia and Kastan, 1998; Levine, 1997)). Normally, p53 is present at low levels in the cell due to its rapid turnover; however, in response to activating signals, p53 becomes stabilized and enriched in the nucleus. p53 is regulated by post-translational modifications including phosphorylation and acetylation events that contribute both to the stabilization of p53 and to the conversion of p53 from a latent to an active transcription factor. Activation of p53 can cause either a cell cycle arrest or apoptosis, responses that are largely mediated by activation of p53-responsive target genes. The product of the p53 target gene MDM2 is involved in p53 ubiquitination and destruction via the proteasome, keeping p53 at low levels in unstressed cell (Haupt et al., 1997; Kubbutat et al., 1997). MDM2 also inhibits p53 by binding to the transactivation domain of p53 (Oliner et al., 1993).

Disruption of the negative regulation of p53 by MDM2 is thought to be critical to stabilizing p53, and phosphorylation of p53 has been thought to contribute to the disruption of p53/MDM2 interaction (Chehab et al., 1999; Shieh et al., 1997; Unger et al., 1999a). Human p53 is phosphorylated on multiple N-terminal residues following DNA damage, including Serines 6, 9, 15, 20, 33, 37, 46 and Threonine 18 (reviewed in (Appella and Anderson, 2001). The physiological role of most of these phosphorylation events has not been clearly defined. It has been shown in vivo that the ATM (ataxia-telangectasia mutated) kinase is important for p53 stabilization, as in cells lacking functional ATM there was a delay in p53 stabilization and reduced Ser15

phosphorylation following ionizing radiation (Siliciano et al., 1997). Data from in vitro systems suggest that the phosphorylation of Ser15 of p53 by ATM may be direct (Banin et al., 1998; Canman et al., 1998). However, the functional consequences of Ser15 phosphorylation are controversial, with reports supporting an effect of Ser15 phosphorylation on the p53/MDM2 interaction (Shieh et al., 1997) and others concluding that Ser15 phosphorylation does not block this interaction (Dumaz and Meek, 1999). Phosphorylation of Ser15 has also been implicated in activating p53 for transcriptional transactivation (Dumaz and Meek, 1999). Effects of Ser15 phosphorylation independent of p53 stabilization may be critical in vivo; cells from mice with germline point mutation of Ser15 exhibited normal p53 stabilization but thymocytes exhibited partially impaired p53-dependent apoptosis (Chao et al, 2003, Sluss et al 2004).

Ser 20 of human p53 is also phosphorylated upon DNA damage (Shieh et al., 1999; Unger et al., 1999a), and this phosphorylation has been reported to be critical for p53 stabilization. Of the sites thought to be phosphorylated upon DNA damage, Ser 20 and Thr 18 (but not Ser15) lie directly in the segment of p53 that interacts with MDM2 (Kussie et al., 1996). Transfection of the p53 Ser20Ala phosphorylation site mutant into various human cell lines completely prevented induction of p53 protein levels in response to gamma or UV radiation (Chehab et al., 1999). Other experiments involving the transfection of exogenous p53 point mutants have also demonstrated defects in p53 stability as well as apoptosis when Ser20 was mutated (Unger et al., 1999a; Unger et al., 1999b). However, similar experiments have shown that multiple N-terminal phosphorylation sites, including Ser15 and Ser20, can be mutated to alanine without dramatic effect on p53 stability (Ashcroft et al., 1999; Blattner et al., 1999).

Interpretation of the data from experiments using ectopically expressed p53 is confounded by possible non-physiological regulation of p53 in this context. That is, the balance between p53 and MDM2, which is likely to be critical for proper p53 regulation, may not be properly achieved when p53 or MDM2 is ectopically expressed. Indeed, in one report in which p53 point mutants were co-transfected with MDM2 the importance of Ser20 mutation for p53 stability differed depending on the amount of p53 transfected (Dumaz et al., 2001). This illustrates the importance of examining p53 phosphorylation sites in vivo at endogenous levels to definitively determine the function and importance of the phosphorylation site. Importantly, while there is ample evidence for phosphorylation of human p53 on Ser20, phosphorylation of the equivalent residue on murine p53 (Ser23) has been less rigorously established due to the lesser quality of phospho-specific antibodies for the mouse.

Chk2 has emerged as a critical regulator of p53 downstream of ATM, and it is phosphorylated and activated by ATM in response to ionizing radiation (Matsuoka et al., 1998; Melchionna et al., 2000). A genetic connection between Chk2 and p53 was revealed by the finding of Chk2 mutations in patients with Li-Fraumeni syndrome (LFS), which predisposes patients to a tumors in multiple tissues, including sarcomas, breast and brain tumors (Bell et al., 1999). LFS is normally associated with germline mutations in p53; however, the patients with Chk2 mutation had wild-type germline p53 (Bell et al., 1999). Studies of murine cells lacking Chk2 indicate that it is important for p53 function. Chk2-/- animals show strong impairment in apoptosis in the irradiated nervous system and thymus (Hirao et al., 2002; Hirao et al., 2000; Takai et al., 2002). In one study, Chk2-/- thymocytes were completely defective for p53 stabilization following DNA

damage (Hirao et al., 2000). However, more recently, germline Chk2 -/- animals were generated that showed a mild defect in p53 stabilization and strong defects in activation of p53 target genes (Takai et al., 2002). Ser20 phosphorylation has been proposed to mediate the effects of Chk2 on p53 induction. Chk2 can phosphorylate Ser 20 of p53 in vitro (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). Transfection of a dominant-negative Chk2 inhibited Ser20 phosphorylation on p53 following DNA damage (Chehab et al., 2000). In vitro, Chk2 expression disrupted preformed complexes of p53 and MDM2 except when Ser20 was mutated to alanine (Chehab et al., 2000). However, Chk2 can phosphorylate multiple sites on p53 in vitro, including Ser15, Thr18 and Ser20 as well as undetermined sites in the C-terminus (Shieh et al., 2000); the sites phosphorylated by Chk2 on p53 in vivo have not been rigorously defined. Also, Chk2 may phosphorylate targets other than p53 itself that help to regulate p53 stability. Finally, recent data demonstrate that Chk2 function is not essential for Ser20 phosphorylation, as p53 can be efficiently phosphorylated on Ser20 in Chk2-/- cells (Jallepalli et al., 2003; Takai et al., 2002). In addition to Chk2, Chk1 (Shieh et al., 1999) and PLK3 (Xie et al., 2001) have been reported to phosphorylate Ser20 in vitro. The specific kinase responsible for Ser 20 phosphorylation in vivo has yet to be determined.

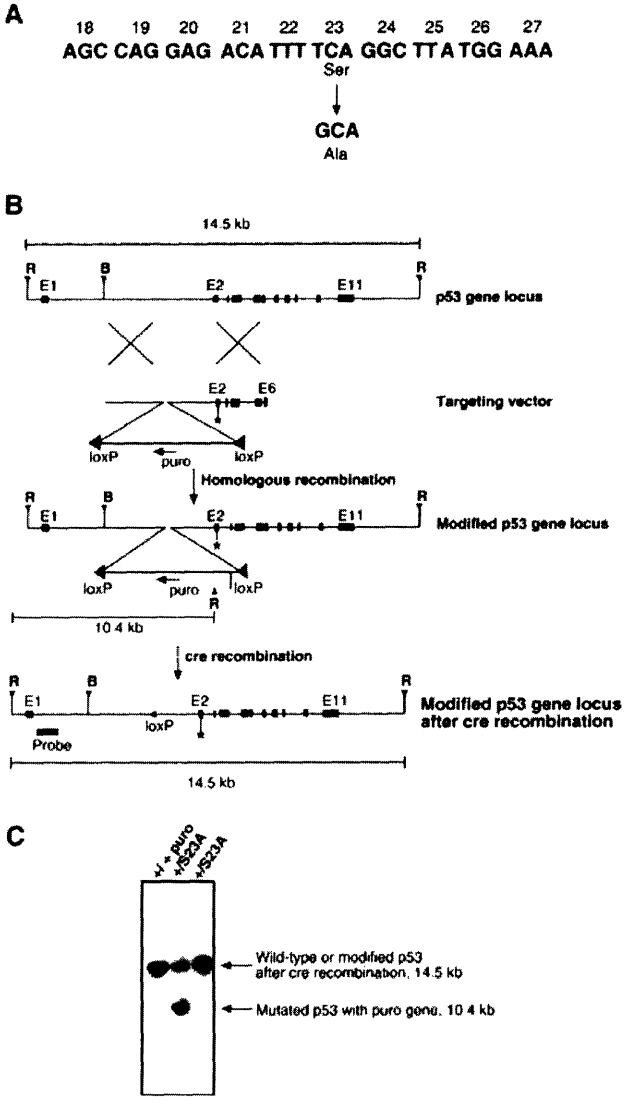
Because of many indications that Ser20 phosphorylation is important for p53 function, we sought to determine the effects of preventing phosphorylation at the murine equivalent of this residue (Ser23). We targeted the endogenous p53 locus and mutated Ser23 to Ala, a residue that cannot be phosphorylated. A similar approach was recently described by Wu et al. (2002), who also targeted the murine p53 locus to generate Ser23 to Ala mutant ES cells. This group also derived mouse embryonic fibroblasts (MEFs) and

thymocytes through blastocyst complementation approaches and concluded from their studies that Ser23 phosphorylation is not important for murine p53 function. Importantly, p53 function was studied under cell culture conditions and germline Ser23 mutant animals were not generated. Here we report the generation of germline Ser23 to Ala mutant mice. Our studies of these mice suggest an important role for ser23 phosphorylation in p53 response to DNA damage in vivo and in tumor suppression.

Results

Generation of mice with p53 S23A mutation

To generate a mouse with the endogenous serine 23 mutated, we used homologous recombination to introduce a mutation into the p53 genomic locus. The targeting construct contained the Ser23 mutation in exon 2 and a puromycin gene flanked by loxP sites in intron 1 (Fig. 1a,b). ES cells were targeted and homologous recombination was confirmed by Southern blotting (Fig. 1c, lane2). Treatment of targeted ES cells by transient transfection with a Cre-expression plasmid led to excision of the puromycin cassette, which was also confirmed by Southern blotting (Figure 1c, lane3). ES cells from four independently targeted ES lines were injected into blastocysts to generate chimeras. Five chimeras were generated from two of the independently targeted ES lines. Four of these chimeras transmitted the mutant allele (termed S23A) to the germline and their progeny were used in the experiments described. S23A heterozygous mutant mice were intercrossed to obtain S23A homozygotes. These animals were born at Mendelian ratios and had no overt phenotypes at birth (similar to p53 null animals). RT-PCR for p53 was performed on MEFs derived from S23A animals and sequencing of the entire p53 open reading frame demonstrated the presence of the targeted mutation and confirmed that no additional mutations had occurred in the coding sequence of the gene (data not shown). Animals described here (and cells obtained from these) are of mixed (129/sv x C57BL/6) background.


A
18 19 20 21 22 23 24 25 26 27
AGC CAG GAG ACA TTT TCA GGC TTA TGG AAA
Ser
GCA
Ala
B
14.5 kb
R
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E2
E11
p53 gene locus
E2
E6
Targeting vector
loxP
puro
loxP
Homologous recombination
Modified p53 gene locus
10.4 kb
cre recombination
Modified p53 gene locus
after cre recombination
Probe
14.5 kb
C
+/+
+ puro
+/523A
+/523A
Wild-type or modified p53
after cre recombination, 14.5 kb
Mutated p53 with puro gene, 10.4 kb

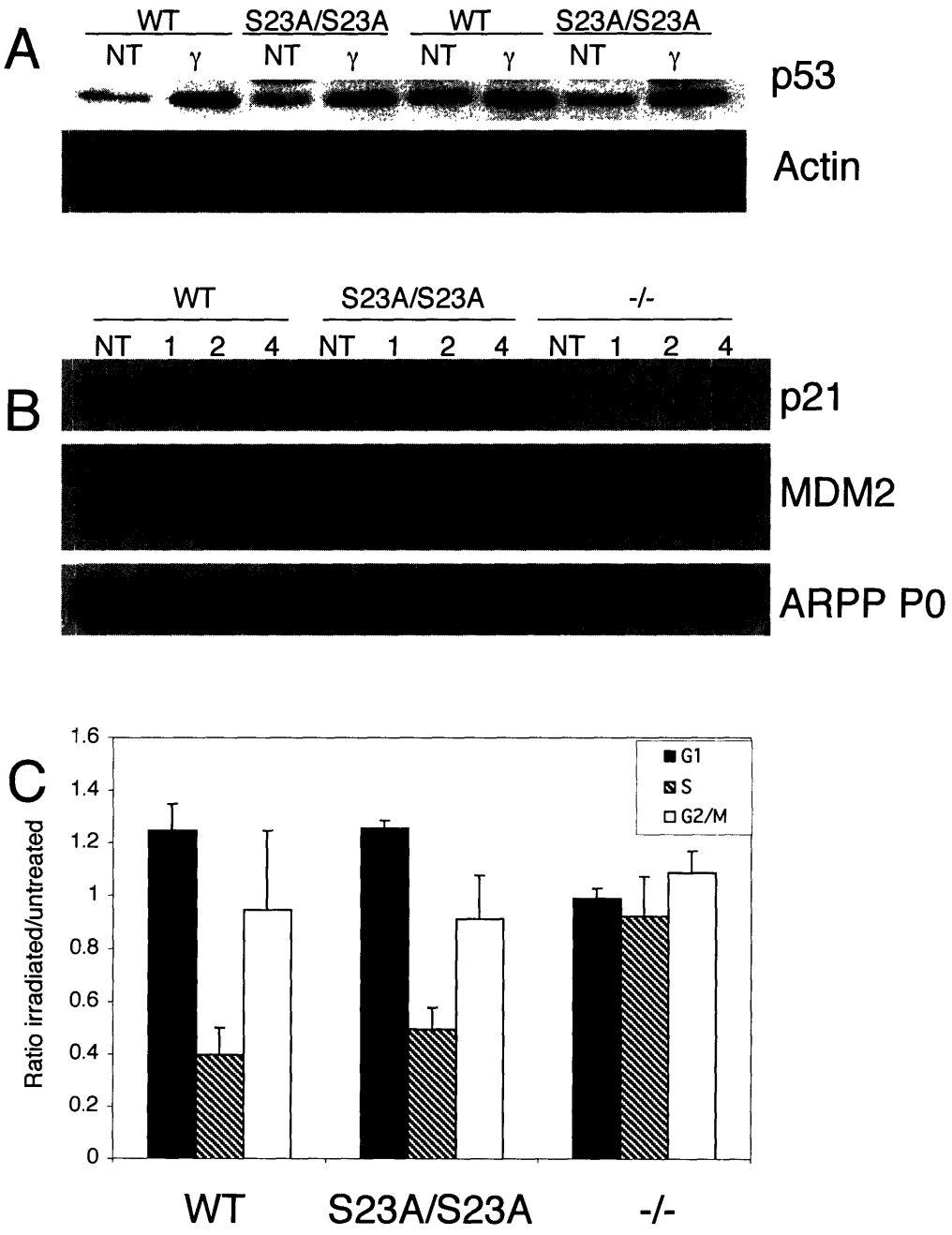
Figure 1. Targeting of endogenous p53 locus to introduce a Ser23 to Ala mutation. A, The S23A mutation that is introduced by a single-nucleotide substitution is represented. B, Schematic representation of targeting strategy. The targeting construct introduces the exon 2 point mutation and also introduces into intron 1 a puromycin cassette in the reverse orientation flanked by loxP (◀) sites, and containing an EcoR1 site. The modified p53 locus following homologous recombination is also shown. Following homologous recombination Cre recombinase was introduced by transient transfection to delete the Puro cassette, leaving a single loxP site. The position of the probe, external to the targeting cassette is shown and the EcoR1-cut sites, internal and external to the cassette are also indicated. Abbreviations used are: R, EcoR1; B, BamH1; puro, puromycin-resistance gene. C. Genotyping of ES cell clones that survived puromycin selection. Southern blot analysis was performed on EcoR1-digested genomic DNA. The probe hybridizes to a 14.5 kb EcoR1 fragment from the unmodified locus (+/+). Correctly targeted heterozygous ES cells surviving puromycin selection also show the 10.4 kb EcoR1 fragment (+/S23Apuro). Following the addition of Cre to targeted ES cell, the Puro cassette is excised (+/S23A).

S23A MEFs stabilize p53 and undergo cell cycle arrest in response to DNA damage

We generated S23A/S23A MEFs from embryonic day 13.5 (E13.5) embryos to determine if Ser23 phosphorylation was required for p53 stabilization. Surprisingly, we found p53 protein levels could clearly be induced in S23A/S23A MEFs following 5 Gy gamma irradiation, although in some experiments at slightly decreased levels compared to wild-type controls (Fig. 2a). Similar results were found with a dose of 10 Gy (not shown). Furthermore, p53 was fully functional in its ability to induce expression of p21 and MDM2 (Fig. 2b). p53 null MEFs are resistant to a G1 arrest in response to gamma irradiation (Kastan et al., 1992), and this effect is in large part mediated by p21 (Brugarolas et al., 1995; Deng et al., 1995). In contrast, S23A/S23A MEFs arrested in G1 with no clear defect compared to wild-type cells when assayed 14 hours (Fig. 2c) or 24 hours (data not shown) following gamma irradiation.

Defective apoptosis in S23A mutant thymocytes

While MEFs respond to gamma irradiation by undergoing p53-dependent cell cycle arrest and not apoptosis, thymocytes undergo apoptosis in response to the same stimulus. p53-null thymocytes are completely resistant to gamma irradiation-induced apoptosis under the experimental conditions assayed (Clarke et al., 1993; Lowe et al., 1993). Thymocyte apoptosis assays were performed on S23A thymocytes to determine if phosphorylation of Ser23 is essential for p53 induction and function in this system. Isolated thymocytes were plated, treated with IR and apoptosis was assessed 6, 12 and 24 hours later. S23A/S23A thymocytes were not completely resistant to IR-induced apoptosis, in contrast to p53 null


A
WT
S23A/S23A
WT
S23A/S23A
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γ
NT
γ
NT
γ
NT
γ
p53
Actin
B
WT
S23A/S23A
-/-
NT 1 2 4 NT 1 2 4 NT 1 2 4
p21
MDM2
ARPP P0
C
Ratio irradiated/untreated
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
G1
S
G2/M
WT
S23A/S23A
-/-

Figure 2. Serine 23 phosphorylation is not required for p53-dependent stabilization or cell cycle arrest in MEFs treated with ionizing radiation. A, Western blot analysis of p53 stabilization 1 hour following treatment with 5Gy IR. Two independent MEF lines are shown. B, Northern blot analyses of the p53 target genes p21 and MDM2 in WT and S23A/S23A MEFs 1, 2 and 4 hours following 5Gy IR. C, Cell cycle analysis of S23A/S23A and control MEFs following gamma irradiation. G1, S and G2/M cell populations were sorted by flow cytometry 14 hours following treatment with 5Gy IR. Numbers are relative to those obtained from unirradiated cells. Data are from 3 experiments and standard deviations are indicated (error bars).

cells. However, they showed an intermediate level of resistance at the 12 and 24 hour time points (Fig. 3a). This partial resistance was also seen at each dose of radiation tested in a dose response experiment (Fig. 3b). It has been previously shown that the induction of apoptosis in thymocytes is sensitive to the levels of p53 induced, as p53 heterozygotes were partially resistant to apoptosis in this assay (Clarke et al., 1993; Lowe et al., 1993). S23A/+ thymocytes showed an intermediate phenotype between +/+ and S23A/S23A thymocytes (Fig. 3a,b) supporting the notion that apoptosis in this assay is sensitive to the dose of p53. In contrast to the resistance following gamma irradiation, no resistance was shown to the p53-independent apoptosis induced by dexamethasone (Fig. 3a,b). To determine if the defect in IR-induced apoptosis correlated with decreased p53 protein induction, we isolated thymocytes, irradiated them under cell culture conditions and performed a time course examining p53 stabilization in this tissue. A modest but clear defect in p53 protein levels induced following irradiation was observed (Fig. 3c). Examination of p53 protein levels in the thymus following whole-body irradiation in vivo revealed an even stronger difference between control and S23A heterozygous or homozygous mutant cells (Fig. 3d). Basal levels of p53 in uninduced thymocytes was low; however, it was apparent that there was decreased basal levels of p53 protein with the Ser23 mutation (Fig. 3d). Importantly, mRNA levels of p53 in the thymus were not changed with Ser23 mutation (not shown). The decreased protein level of p53 induced in the thymus following IR, either in vitro or in vivo, suggests that the apoptotic defect is due to decreased p53 stability when Ser23 is mutated. To investigate this further, we examined p53 protein stability following DNA damage. The protein synthesis inhibitor cycloheximide was added to isolated thymocytes 2 hours following treatment with 5 Gy

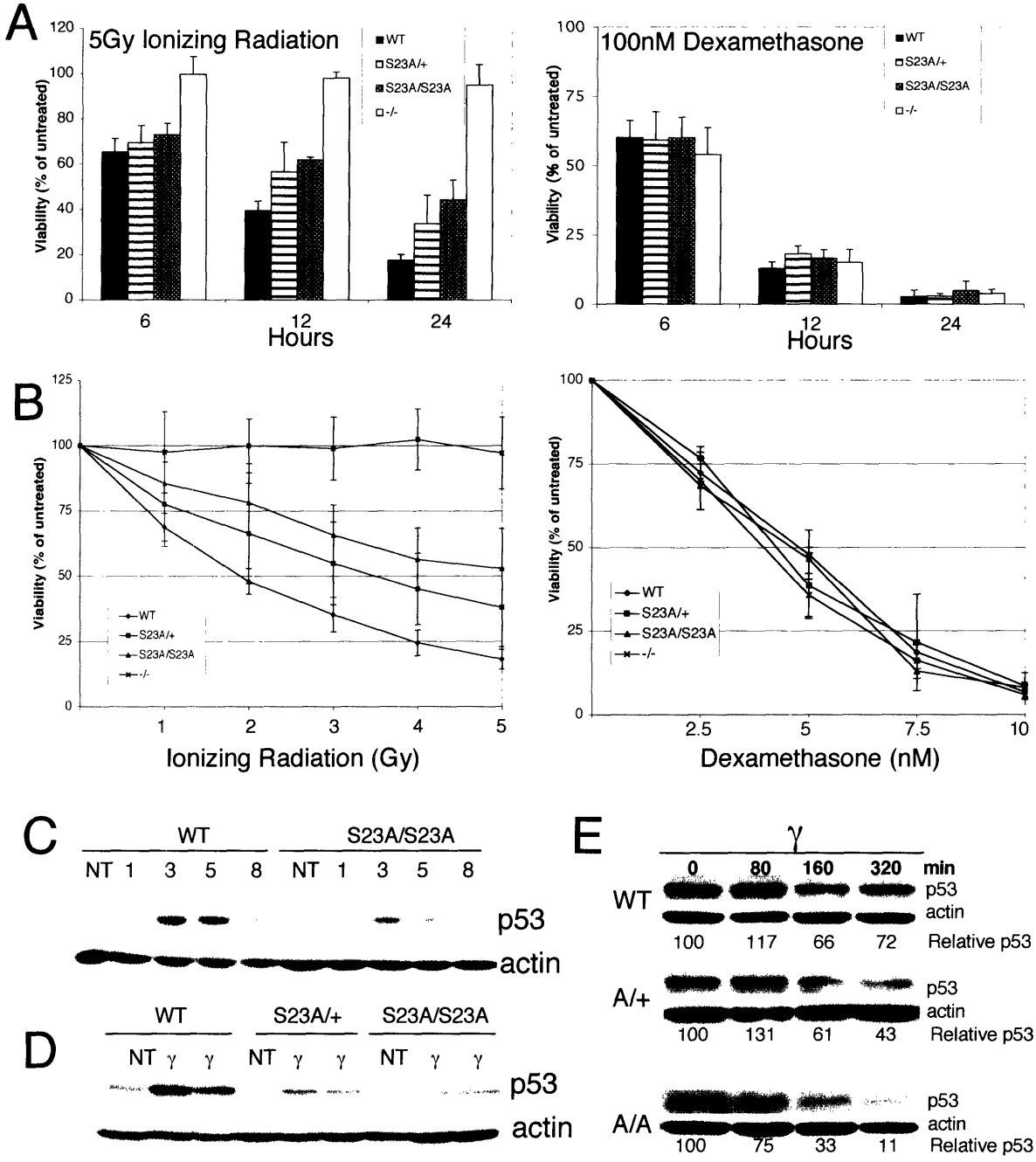

A
5Gy Ionizing Radiation
WT
S23A/+
S23A/S23A
-/-
Viability (% of untreated)
Hours
100nM Dexamethasone
B
Ionizing Radiation (Gy)
Dexamethasone (nM)
C
WT
S23A/S23A
NT 1 3 5 8 NT 1 3 5 8
p53
actin
D
WT
S23A/+
S23A/S23A
NT γ γ NT γ γ NT γ γ
E
γ
0 80 160 320 min
WT
A/+
A/A
p53
actin
100 117 66 72 Relative p53 level
100 131 61 43 Relative p53 level
100 75 33 11 Relative p53 level

Figure 3. Decreased apoptosis and p53 stabilization in S23A mutant thymocytes following DNA damage. A, Flow-cytometric analysis of apoptosis in isolated thymocytes irradiated with 5 Gy or treated with 100nM dexamethasone. Cells were plated at 1 x 10^6 cells/mL prior to treatments, and apoptosis was assessed at various time points using two-color cytometry following staining of cells with propidium iodide (PI) and annexin-FITC. Viable cells were both annexin and PI negative. All values were expressed relative to the viability in untreated samples, which was 65-70% at 24 hours and did not differ between the genotypes examined. For WT, S23A/+ and S23A/S23A data, littermates were used in each experiment. B, Thymocyte viability 24 hours following varying doses of ionizing radiation, or dexamethasone. For all apoptosis experiments, values represent the averages of 5 experiments, with error bars indicating the standard deviation. C, p53 protein levels were determined by immunoblot in isolated thymocytes irradiated in vitro with 5 Gy and then cultured for 1,3,5, or 8 hours. D, p53 protein levels were determined by immunoblot in thymus extracts from WT, S23A/+ or S23A/S23A mice that had been irradiated with 5 Gy five hours before thymus collection. One untreated and two irradiated samples of each genotype are shown. E, p53 stability in WT, S23A/+ or S23A/S23A isolated irradiated thymocytes. Cycloheximide was added to inhibit protein synthesis 2 hours following 5Gy irradiation. Band intensities were normalized to actin levels and levels relative to the T=0 point for each genotype are shown. This figure is representative of four experiments.

gamma radiation, and p53 protein levels were then followed over time. As shown in Figure 3e, the half-life of p53 in S23A/S23A cells was significantly reduced compared to wild-type p53. S23A/+ cells exhibited an intermediate phenotype. Thus, S23A mutation reduces p53 stability following DNA damage in thymocytes.

We also examined apoptosis in the splenic white pulp, which contains both B- and T-lymphocytes and is a site of p53-dependent apoptosis following irradiation in vivo (Komarova et al, 2000). TUNEL assays showed that 6-hours following irradiation with 5Gy in vivo, there were high levels of apoptosis in both the control and S23A/S23A splenic white pulp without dramatic differences between these genotypes (Fig. 4a). By 18-hours post-irradiation, overall levels of apoptosis were lower in both the wild-type and S23A/A spleens, facilitating quantification. There was a 3.6-fold decrease in white-pulp apoptosis in S23A/S23A animals versus wild-type controls ($p<0.001$; Student's T-test) (Fig 4a,b). We performed FACS analyses using the B-cell marker B220 to quantify depletion of splenic B-lymphocytes 24-hours following irradiation in vivo. As shown in Figure 4c, while the proportion of B220 positive splenocytes in untreated wild-type and S23A/S23A animals was similar, by 24 hours following irradiation the S23A/S23A animals were significantly resistant to loss of the B-cell population ($p=0.025$; Student's T-test). Thus, Ser23 mutation leads to partial resistance of splenic B-cells to irradiation-induced apoptosis.

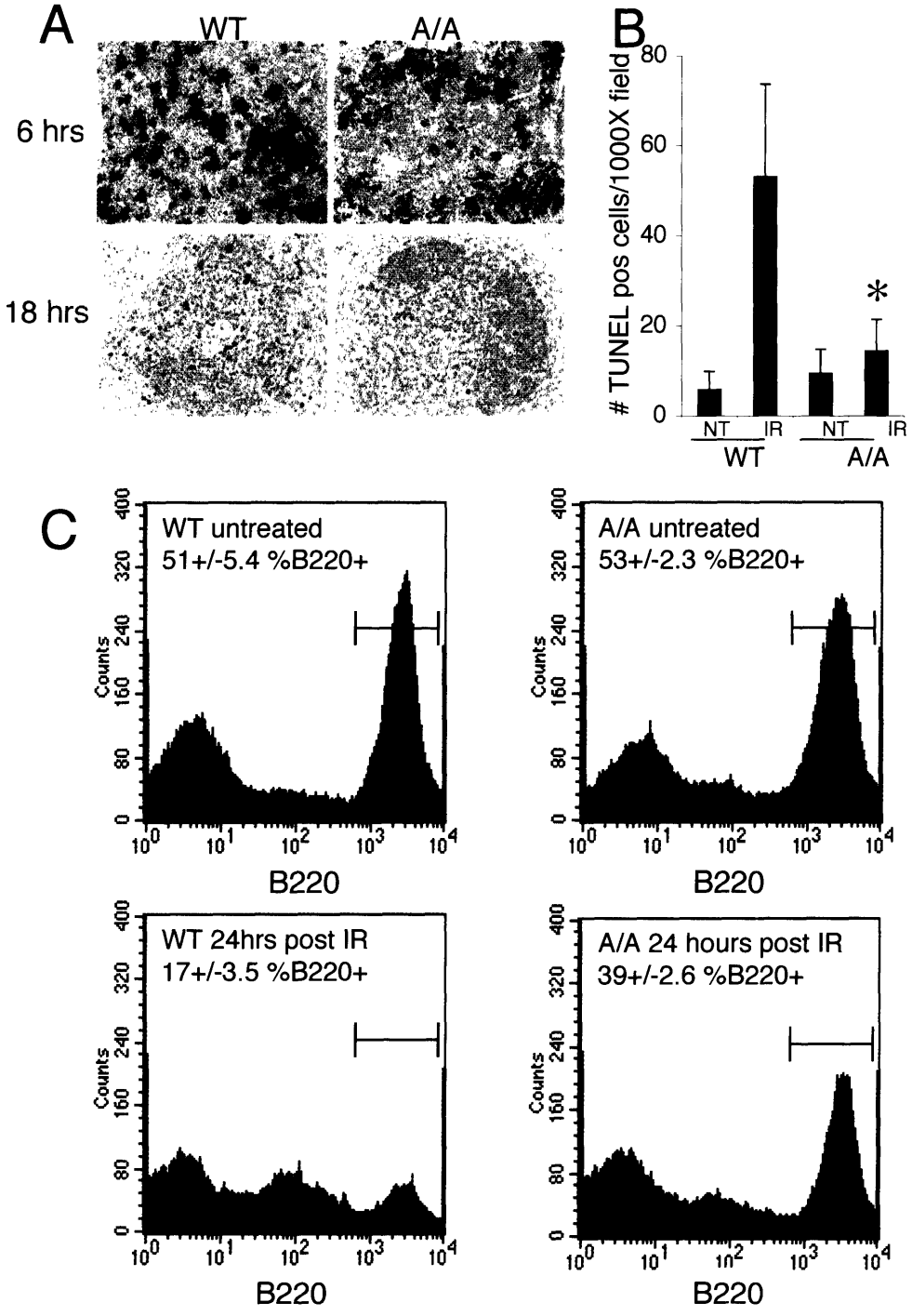

A
WT
A/A
6 hrs
18 hrs
B
TUNEL pos cells/1000X field
0
20
40
60
80
NT
IR
NT
IR
WT
A/A
*
C
WT untreated
51+/-5.4 %B220+
A/A untreated
53+/-2.3 %B220+
WT 24hrs post IR
17+/-3.5 %B220+
A/A 24 hours post IR
39+/-2.6 %B220+
Counts
B220

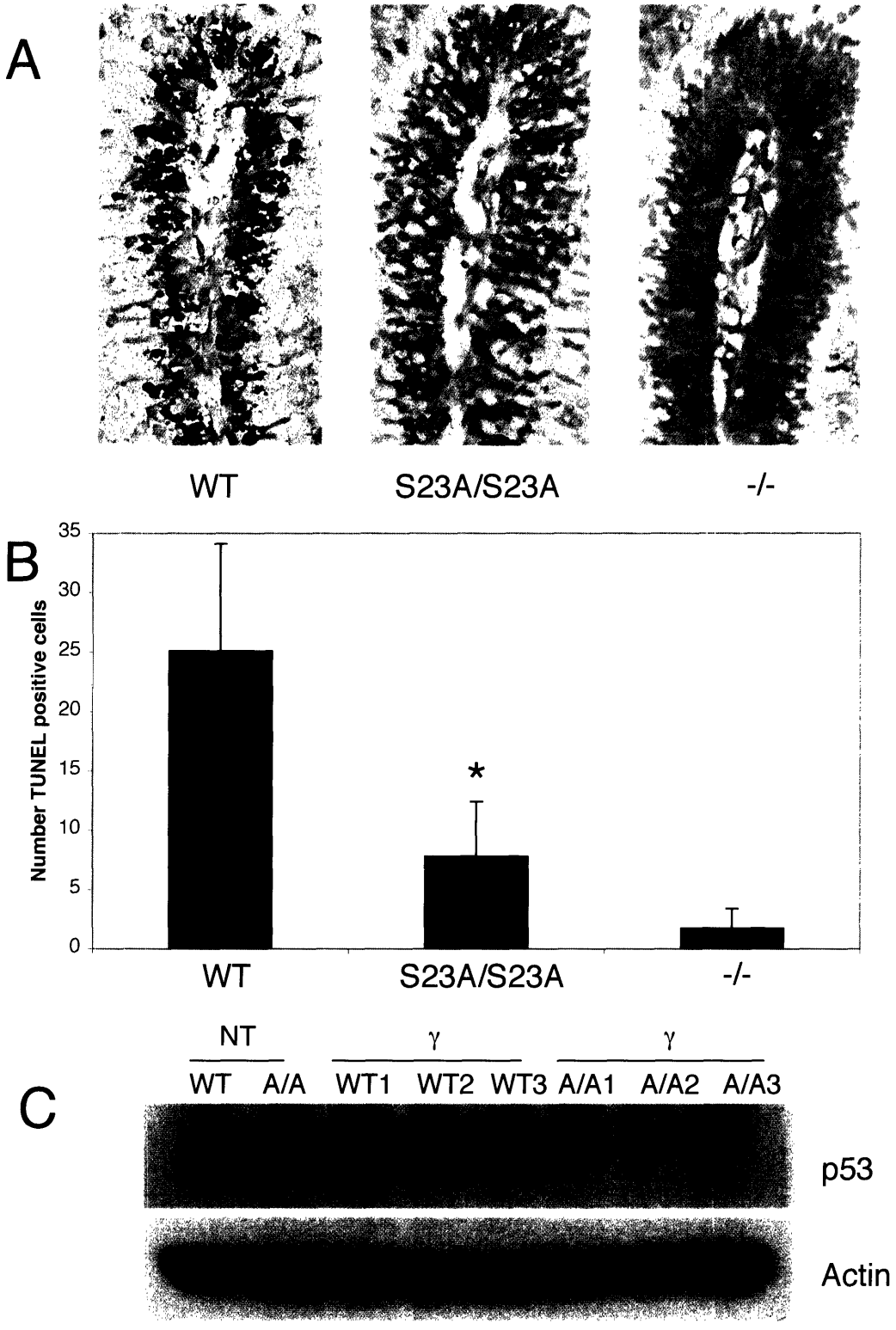
Figure 4. Decreased apoptosis in splenic B-cells of S23A/S23A mice exposed to gamma radiation in vivo. A. TUNEL staining to assess apoptosis in the splenic white pulp 6 hours, and 18 hours following gamma irradiation in S23A/S23A or control animals. B. Quantification of TUNEL-positive cells in the splenic white pulp at 18-hour time point post- irradiation or in untreated wild-type or S23A/S23A animals. C. FACs analysis of B220 positivity in wild-type and S23A/S23A splenocytes 24 hours post 5 Gy irradiation or from untreated controls in vivo. Percentage of B220 positive cells +/- S.D. from 3 independent experiments is indicated.

S23A developing cerebellum is resistant to DNA damage-induced apoptosis

We next examined effects of S23A mutation on p53 function in another cell population that undergoes p53-dependent apoptosis in vivo. We focused our analysis on gamma radiation-induced apoptosis in the external granule layer in the developing cerebellum, a neuronal population of cells that undergo apoptosis in both a p53 and ATM-dependent manner (Herzog et al., 1998). 5 days old mice (P5) were irradiated and we assessed apoptosis 18 hours later by TUNEL analysis. As shown in Figure 5a, significantly decreased CNS apoptosis in S23A/S23A animals compared to wild type was observed. Quantification of TUNEL positive nuclei revealed that there was 3.7 fold more apoptosis in the wild type compared to S23A/S23A cerebellum (Fig. 5b). As with thymocytes, the phenotype seen with the point mutant did not recapitulate the degree of resistance observed in p53 null mice (Fig. 5a,b). To determine if the defect in apoptosis is due to a defect in p53 stability, we irradiated P5 S23A/S23A, wild-type or p53 null mice and collected the cerebellum 3 hours later to examine p53 levels. p53 protein levels were strikingly decreased in the S23A/S23A samples (Fig. 5c).

Tumorigenesis in S23A/S23A animals

We examined whether mutation of Ser23 would contribute to tumor formation by aging cohorts of S23A/S23A and wild-type animals. p53-/- animals die between 4 and 6 months, usually due to thymic lymphoblastic T-cell lymphomas and sarcomas (Donehower et al., 1992; Donehower et al., 1995; Jacks et al., 1994), S23A/S23A mice lived to a much longer median survival time of 444 days (Fig 6). Necropsies and


A
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B
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NT
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γ
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WT1
WT2
WT3
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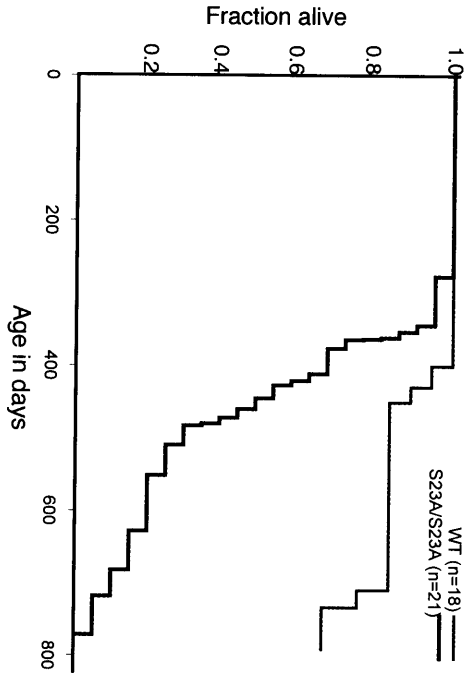
A/A2
A/A3
p53
Actin

Figure 5. Decreased p53 levels and apoptosis in developing cerebellum of S23A/S23A mice exposed to gamma radiation. A, TUNEL staining to assess apoptosis in the external granule layer of P5 developing cerebellum collected 18 hours following 4Gy of gamma radiation. B, Quantification of apoptosis in external granule layer cells. TUNEL positive cells were quantified in equivalent areas. Data are from 8 S23A/S23A animals, 8 WT animals and 4 p53 null animals. S23A/S23A and WT mice used in this study were littermates. * p=0.001 comparing WT to S23A/S23A C, Western blot shows significantly decreased p53 protein levels in S23A/S23A irradiated cerebellum. Postnatal day 5 (P5) mice were treated with 4Gy IR and their cerebellum was collected 3 hours post irradiation. Three wild-type and three S23A/S23A irradiated samples are shown along with untreated controls.


Fraction alive
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400
600
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Age in days
WT (n=18)
S23A/S23A (n=21)

Figure 6. Survival of S23A mutant mice. Kaplan Meier analysis of tumor-free survival in wild-type and S23A/S23A mice. Animals were sacrificed when moribund, or if tumor burden was apparent.

histopathological analyses were performed on moribund mice. Surprisingly, none of these animals developed thymic T-cell lymphoblastic lymphoma. In contrast, a wide spectrum of B-cell lineage tumors were found in S23A/S23A animals, classified based on histological criteria as follicular, diffuse large cell and splenic marginal zone lymphomas, as well as plasmacytomas (Fig 7a-e, Table 1). The most common of these tumors was follicular lymphoma. Mice with this category of tumor typically had predominantly nodal disease (often involving the mesenteric lymph node), with less involvement of the spleen. Extranodal tumor involvement in tissues such as kidney and liver was often found. Most lymph nodes affected by these tumors had a vaguely nodular architecture. In some cases the nodularity was highlighted by immunostaining for plasma cells that located at the periphery of the nodules (Fig 7a). The infiltrate was composed predominantly of small, irregular lymphocytes (centrocytes) with a variable, but typically small, fraction of larger nucleolated lymphcoytes (centroblasts) (Fig 7b). Occasional tumors had higher mitotic rates and increased fractions of large cells. In some cases plasma cells were abundant both within and outside of the infiltrate of lymphoma cells. In the spleen, lymphoma cells largely replaced the white pulp. Follicular lymphomas were positive for the B-cell marker B220 (not shown) and exhibited clonal or oligoclonal IgJ_H rearrangements confirming their B-cell origin (Fig. 7g). We suggest that the plasmacytic cells, which were often atypical in morphology are part of the clonal tumor. However, we cannot rule out the possibility that the plasmacytic component is a separate lesion as other animals without lymphoma also exhibited plasmacytic hyperplasia. In one animal, plasmacellular proliferation and morphology was overtly cytologically malignant, with extensive invasion of extranodal sites (Fig. 7e). This tumor was classified as a malignant

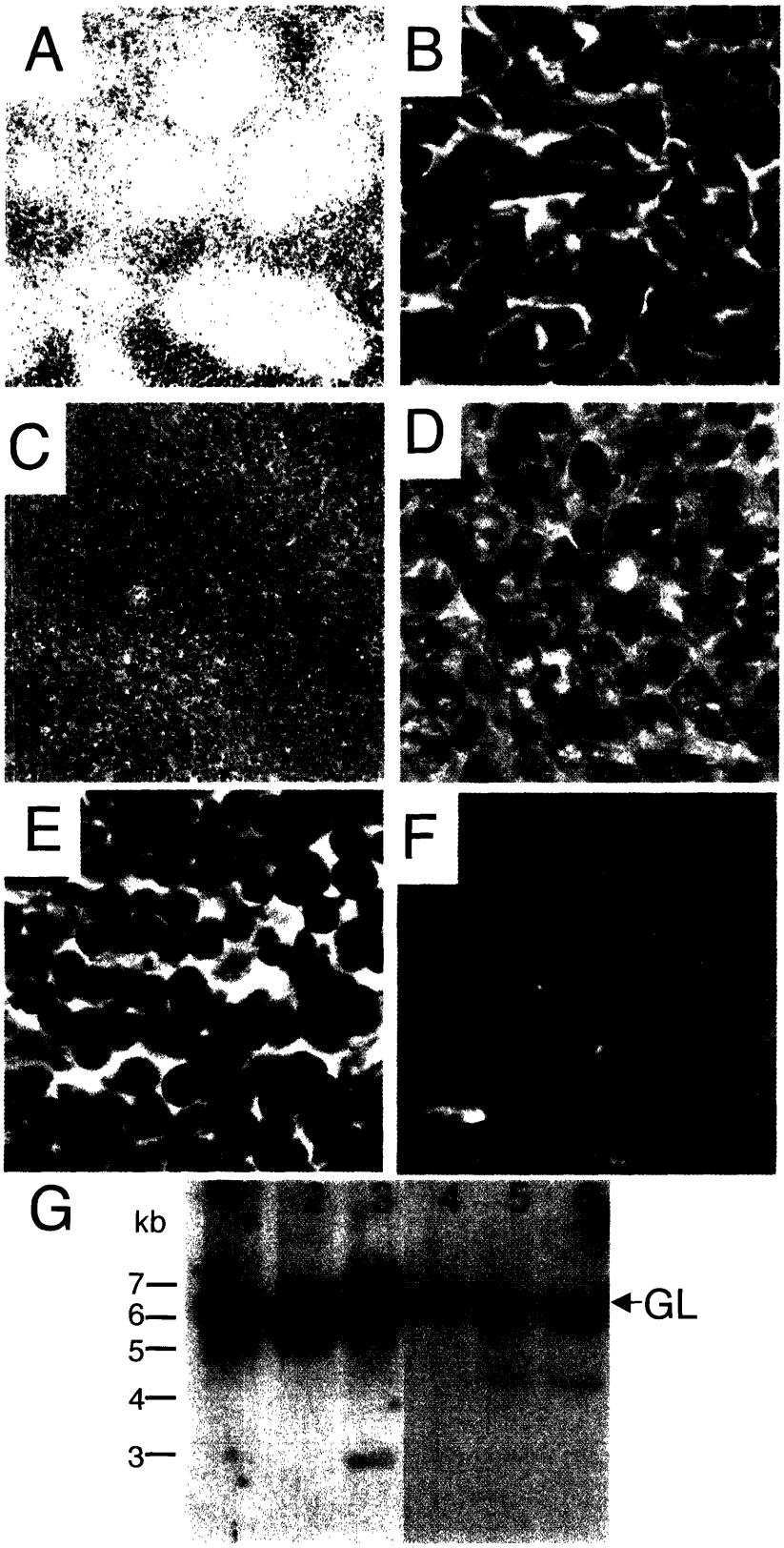
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Figure 7. B-cell tumors in S23A mutant mice. A) Plasma cell immunostaining (anti-Ig kappa light chain) of a follicular lymphoma at 40X showing nodular tumor architecture. B) Follicular lymphoma (H+E 1000X) C) Splenic marginal zone lymphoma (MZL) showing expanded marginal zone with pale eosinophilic cells (H+E 200X) D) High power view of splenic MZL showing the appearance of cells with plasmacytic morphology (H+E 1000X) E) Malignant plasmacytoma (H+E 1000X) F) Histiocytic sarcoma (H+E 1000X) G) Southern blot analysis of immunoglobulin heavy chain recombination. DNA from S23A/S23A tumors (lanes 2-6) or wild-type lymph nodes (lane 1) was digested with EcoR1 and hybridized with a JH region probe. 6.2 kb germline band is indicated (GL). 1. Normal lymph node from wild-type mouse 2. Follicular lymphoma. 3. Follicular lymphoma. 4. Histiocytic sarcoma. 5, 6 Follicular lymphoma in different lymph nodes from same S23A/S23A animal. B-cell tumors in lanes 2,3, 5 and 6 show one or two novel bands indicating tumors are monoclonal with one or both JH alleles rearranged, or biclonal.

plasmacytoma (Table 1). Spleens involved by marginal zone lymphoma had a largely preserved archictecture with the periarteriolar lymphoid area cuffed by small to intermediate sized lymphocytes with oblong to slightly folded nuclei and a moderate amount of pale cytoplasm. Numerous plasma cells were located at the periphery of the infiltrate, often extending into splenic the red pulp (Fig. 7c,d). Two animals had histiocytic sarcomas, which stained positively for the macrophage marker F480 (not shown) and did not exhibit clonal IgJ_H rearrangement (Fig. 7f,g). We note that both of these cases of macrophage tumors occurred in a background of splenic red pulp hyperplasia, including the myeloid, erythroid and megakaryocytic lineages, suggesting that this hyperplasia may be a pre-malignant lesion. Marked red pulp hyperplasia without histiocytic tumors was also observed in four other S23A/S23A animals that did not have lymphomas. Other non-hematopoietic tumors were also observed in S23A/S23A animals (Table 1). During the course of the study, five wild-type mice died, one due to a hemangioblastoma and another with a hepatoma. Our tumorigenesis study indicates that S23A/S23A animals are prone to tumor development in a cell-type specific manner.

Discussion

We demonstrate here that mutating Ser23 to alanine affects both p53 stabilization and function. Mutant thymocytes were partially defective in p53-dependent apoptosis in response to ionizing radiation, and strong resistance was observed in the developing cerebellum. These data show in vivo the importance of this residue for p53 function and have helped define the pathway leading to IR-induced apoptosis in these tissues. Our finding that S23A/S23A animals are tumor prone suggests that Ser23 phosphorylation is

Table 1 Histological classification of spontaneous tumors in S23A/S23A mice

Tumor type	Number of cases (%)
B-cell lineage tumors:	13 (62)
Follicular lymphoma	7 (33)
Splenic Marginal Zone Lymphoma	2 (9.5)
Diffuse Large B-Cell Lymphoma	2 (9.5)
Plasmacytoma – well-differentiated	1 (4.8)
Malignant Plasmacytoma – moderately differentiated	1 (4.8)
Other tumors:	
Histiocytic Sarcoma	2 (9.5)
Hemangiosarcoma	1 (4.8)
Lung Adenoma	1 (4.8)
Lung Adenocarcinoma	1 (4.8)
Hepatoma	1 (4.8)
Hair Matrix tumor	1 (4.8)

important for the tumor suppressor function of p53. This is the first report of a p53 phosphorylation site mouse mutant that is prone to spontaneous tumorigenesis.

A previous report of p53 S23A mutant function in targeted murine cells concluded that Ser23 phosphorylation is not important for p53 stabilization and function in various cell types, including ES cells, MEFs and thymocytes (Wu et al., 2002). While we have not found strong effects of this mutation on p53 function in MEFs, we observe significant defects in p53 stabilization and apoptosis in irradiated thymocytes. The study by Wu et al., (2002) was different in that germline S23A/S23A animals were not generated, and the thymocytes they studied were generated through a RAG2-deficient blastocyst complementation approach. In addition, this group studied S23A/- cells in comparison to p53+/- controls, while we compared S23A/S23A and S23A/+ cells to wild type controls. Because p53+/- cells exhibit significant resistance to apoptosis compared to wild-type cells (Clarke et al., 1993; Lowe et al., 1993), it is possible that differences in thymocyte apoptosis and p53 stabilization were not found due to the smaller window these authors may have had for detecting a subtle difference. Also, we found the strongest effects of this mutation following irradiation in vivo. This was especially true in the irradiated developing cerebellum, which this group was unable to study, and in tumorigenesis.

Ser23 mutation did not have major effects on p53 stabilization under some conditions. For example, in S23A/S23A MEFs, IR-induced p53 stabilization was not dramatically reduced and p53-dependent cell cycle arrest was intact. This is in contrast to the strong defect in levels of p53 protein induced in the thymus and cerebellum irradiated in vivo. Tissue culture stress, including high oxygen concentrations, may allow p53 to be

sensitized to IR-induced stabilization even in the absence of Ser 23 phosphorylation. Indeed, in the thymus irradiated in vivo, a stronger defect in induction was observed compared to thymocytes irradiated in vitro (Fig.3c,d).

The defect in apoptosis in the irradiated Ser23 mutant thymus and CNS clearly correlates with defective p53 induction. The Chk2 kinase has been implicated as a Ser23 kinase in response to ionizing radiation and Chk2-/- mice are defective for p53-dependent apoptosis in the irradiated thymus and CNS (Hirao et al., 2002; Hirao et al., 2000; Takai et al., 2002). Much stronger resistance to thymocyte apoptosis was found in Chk2-/- cells compared to our findings in S23A/S23A thymocytes, indicating that in these cells Chk2 has effects on p53 that are not mediated by Ser23 phosphorylation. Data from targeted Chk2-/- cells suggested that Chk2-/- thymocytes are defective for p53 stabilization following IR; however, the extent of this effect differed from mild (Takai et al., 2002) to a complete lack of induction (Hirao et al., 2000). Whether the defective apoptosis in Chk2-/- animals is mediated through effects on p53 stabilization is not clear, as defects in transactivation of p53 target genes in Chk2-/- cells has been reported in one study (Takai et al., 2002). In vitro, Chk2 has been reported to phosphorylate multiple sites on p53, including Ser15, Thr18, and Ser37 (Shieh et al., 2000). Chk2 can also phosphorylate undetermined C-terminal sites of p53 mutants lacking the N-terminus which could mediate effects of Chk2 independent of p53 stabilization (Shieh et al., 2000). It is also possible that Chk2 could affect p53 indirectly. It is apparent that p53 can be efficiently phosphorylated on Ser 20/23 in Chk2-/- cells (Jallepalli et al., 2003; Takai et al., 2002), and it remains to be determined how much of the effects of S23 mutation are due to an

inability of Chk2 to phosphorylate p53 on this residue. It will be important to determine if older Chk2-/- animals show a tumor spectrum similar to S23A/S23A mice.

It was striking that S23A/S23A mice developed B-cell tumors but not T-cell lymphomas or frequent sarcomas. B-cell tumors have been observed in p53-/- mice (Donehower et al., 1995; Ward et al., 1999); however, fatal thymic lymphoblastic T-cell lymphomas and sarcomas may have limited the development and progression of B-cell tumors in p53-/- animals. It is possible that there is a different threshold for the amount of p53 signaling needed to suppress tumorigenesis in different tissues. For example, thymocytes may be sensitized to p53-dependent apoptosis such that even low levels of p53 are sufficient to induce apoptosis. Indeed, in thymocytes, while there was resistance to apoptosis, a time course showed that S23A/S23A thymocytes do eventually die in response to radiation while at those doses, p53-/- cells were completely resistant (Fig. 3a,b). In response to tumor initiating events cells in other tissues may need a higher degree of p53 signaling for apoptosis and tumor suppression. Alternatively, the upstream pathways to p53 activation may differ in different cell types in response to different stresses such that Ser20/23 phosphorylation is critical in some but not other situations. The specific role of Ser23 in tumor suppression in B-cells could be due to impaired apoptosis in Ser23 mutant B-cells, as splenic B-cells showed partial resistance to irradiation induced apoptosis. p53 function has also been implicated in B-cell proliferation and maturation (Shaulsky et al, 1991, Aloni-Grinstein et al, 1995, Shick et al, 1997). A close examination of the role of the p53/Ser23 pathway in the B-cell differentiation program, and study of differentiation marker expression in the B-cell lineage tumors of Ser23 mutant mice is warranted. Because we observed a long latency to

tumorigenesis in these animals, it is likely that cells needed time to acquire genetic changes to develop into B-cell tumors. It will also be important to identify the genetic changes that lead to the B-cell lymphomas in this model, and to investigate how mutation of the Ser23/p53 pathway cooperates with such events. Moreover, based on these results the role of p53 pathway mutations in human B-cell lymphoma bears further examination.

While p53 undergoes numerous phosphorylation events, the physiological role of these modifications has been difficult to assess. We have shown that p53 Ser23 phosphorylation is critical for p53 stabilization in response to ionizing radiation in vivo, and for tumor suppression. In contrast Ser18 phosphorylation in mice is not required for tumor suppression or, in thymocytes, for p53 stabilization, but may have important roles in apoptosis and/or p53 target gene induction (Chao et al., 2003, Sluss et al. 2004). Bruins et al (in press) find that Ser389, a site phosphorylated after UV irradiation, is important for UV-induced p53 function, and germline Ser389 mutants showed increased sensitivity to UV-induced skin tumors. Collectively, these data demonstrate the importance of generating germline phosphorylation site mutants to dissect the roles of specific p53 phosphorylation events in vivo.

These and other data suggest a model in which in response to ionizing radiation, p53 is stabilized via phosphorylation of Ser20/23. This may be mediated by Chk2, consistent with defective p53 stabilization in Chk2-/- cells, or other Ser23 kinases may be critical. Identification of the pathway upstream of Ser23 phosphorylation may lead to the discovery of genes that could be mutated in tumors in which p53 itself is intact. Given the importance of the p53 pathway in tumor development and the response of cells to

chemotherapy and other DNA damaging agents, it is critical to fully elucidate this pathway.

Material and Methods

Construction of Targeting Vector and generation of S23A mutant mice.

Fragments of mouse genomic p53 sequence extending from intron 1 through exon 6 were cloned into the vector pBSKII. Ser23 is encoded in exon 2 and the single base pair mutation leading to an Ala substitution was introduced by site-directed mutagenesis using the QuikChange kit (Stratagene). A puromycin resistance gene driven by the PGK promoter and flanked by loxP sites (pBS.DAT-LoxStop) was introduced into an Xho1 site in intron 1. p53 exons and intron/exon boundaries in the targeting vector were sequenced to confirm that no additional, unexpected mutations were introduced during the cloning. The targeting vector was linearized with Not1 and electroporated into J1 ES cells (derived from strain 129/sv) before being selected for resistance to puromycin. Homologous recombination was confirmed by Southern blot with EcoR1-digested genomic DNA. The Southern probe was external to the targeting vector and was derived from a Bsm1 digest of a fragment of genomic DNA from intron1. GFP-Cre was transiently transfected into ES cells to excise the puro cassette leaving a single loxP site in intron1. Excision of the puro cassette was confirmed by Southern blot. Heterozygous ES cells were injected into C57BL/6 blastocysts that were then implanted into pseudopregnant CD1 females essentially as described (Bradley 1987). The p53 cDNA sequence from S23A/S23A mice was determined by RT-PCR on RNA isolated from 3 independent MEF lines generated from E13.5 embryos using standard techniques. In each

of the lines, the sequence was the same as wild-type, except for the single T -G base substitution targeted to exon 2. All animals described are on a mixed 129/Sv x C57BL/6 background. PCR genotyping was based on the presence of a single loxP site remaining in intron 1 of the correctly targeted locus. The following primers flanking the remaining loxP site were used: dt020200.1 5'agcctgcctagcttcctcagg3' and dt011200.3 5'cttggagacatagccacactg3'. These primers amplify a 540bp mutant band in the presence of a single loxP site, and a 420bp wild type band.

Western blot analysis

For MEF or thymocyte experiments, cells were plated in DMEM supplemented with 10% FCS, L-glutamine (2mM) and antibiotics. Cells were irradiated using a GammaCell40 with 137Cs source. Cells were lysed in lysis buffer (100mM NaCl, 100mM Tris pH 8.0, 1% NP-40, Complete protease inhibitor cocktail (Roche), sodium flouride (10mM), sodium vanadate (1mM)). For in vivo thymus Western blots, whole mice were irradiated with a dose of 5 Gy and five hours later the thymus was collected for protein extraction. For half-life experiments, thymocytes were suspended at 10 million cells/mL. 40μg/mL cyclohexamide was added two hours post-irradiation and samples collected for Western analysis at various time points afterwards. For cerebellum Western blots, newborn mice five days after birth were irradiated with 4 Gy and three hours later, the cerebellum was dissected and frozen on dry ice before being lysed in lysis buffer. Following SDS-PAGE, proteins were transferred to PVDF membranes (Immobilon P, Millipore). Membranes were blocked in 5% nonfat dry milk in TBST. For p53 Western blots on MEFs, p53 Ab-3 (Oncogene Research Products), was used at a 1/1000 dilution. For p53

Western blots on thymus tissue and cerebellum tissue, the sheep polyclonal antibody Ab-7 (Oncogene Research Products) was used at a 1/2500 dilution, or the rabbit polyclonal antibody FL393 (Santa Cruz) at 1/1000 dilution. Blots were stripped and reprobed for actin (Santa Cruz) at a 1/1000 dilution. Enhanced chemiluminescence was used for signal detection (ECL Plus, Amersham) and blots were exposed to film (X-OMAT). For thymocyte half-life experiments, band intensities were quantified by scanning densitometry using NIH Image software.

Northern blot analysis

Exponentially growing cells were plated at a density of 1 x 10^6cells/ 10cm dish, and treated 24 hours later with 5Gy IR. At various time points cells were lysed directly on the plate with TRIZOL reagent (Gibco BRL) and RNA was isolated following the manufacturers protocol. RNA was denatured and run on a 1% agarose in 1X MOPS with 6% formaldehyde and transferred to a Hybond N membrane (Amersham). The membrane was crosslinked using a UV Stratalinker (Stratagene) and hybridized with a 32P labeled probe. Probes were made to MDM2, p21 and, p53 and ARPP P0 cDNAs using the Prime-It II random primer labeling kit (Stratagene). Blots were hybridized in ExpressHyb (Stratagene) solution at 65° C.

Cell cycle analysis.

Cells were plated at 8 * 10^5 cells/10cM plate and irradiated with 5Gy IR 24 hours later. Analysis of samples was performed 14 hours or 24 hours after IR, as described

(Brugarolas et al., 1998). Samples were processed with a FACScan (Becton Dickinson). Data analysis was done using ModFit LT software (Becton Dickinson).

Thymocyte apoptosis assay

Fresh thymocytes were isolated from 5 to 8 week old mice and plated at a density of 1 x 10^6 cells/mL and then incubated at 37 °C. For time course experiments, cells were treated at T=0 with either 100nM dexamethasone or with 5 Gy of IR. For Dose/Response curves, thymocytes were also plated a 1 x 10^6 cells/mL, treated at T=0 and collected for apoptosis analysis 24 hours later. Apoptosis was assessed using annexin-FITC (Becton Dickinson) and propidium iodide (50μg/mL) staining of cells followed by two-color cytometry using a FACScan (Becton Dickinson) and CellQuest software (Becton Dickinson). Cells scored as viable were those that were negative in staining for both annexin and PI. All data were normalized to the viable fraction in the untreated cells at the given time point.

In vivo analysis of apoptosis

For CNS apoptosis studies, S23A/S23A or wild-type littermates at were irradiated 5 day after birth with 4 Gy. Mice were sacrificed 18 hours later at which time when their brains were removed and fixed in 10% formalin (3.7% formaldehyde solution in PBS) for 24 hours. For analysis of apoptosis in the adult spleen, 6-8 week old animals were irradiated with 5 Gy and sacrificed 24 hours later. Spleens were fixed in 10%formalin for 24 hours. Tissues were processed and embedded in paraffin blocks. Sagittal sections were cut at 4μm. Tdt dUTP-biotin Nick End Labeling (TUNEL) assays were performed to assess

apoptosis. For quantification of CNS apoptosis, TUNEL positive nuclei in equivalent areas of the cerebellar external granule layer of S23A/S23A and control animals were counted. For quantification of B-cell depletion following DNA damage, animals were irradiated with 5Gy 24 hours before collection of spleens and isolation of splenocytes. Splenocytes were stained with anti-B220 coupled to flourescein-isothiocyanate (Pharmingen), using standard protocols and subject to fluorescence activated cell sorting analysis.

Tumor Analysis

S23A/S23A and wild-type controls were aged. Mice were sacrificed when moribund, or if visible tumor burden was apparent. Inner organs were fixed in 10% neutral buffered formalin. Bones were fixed in Bouins fixative. Tissue was embedded in paraffin and 4 μm sections were cut and stained with hematoxylin and eosin for pathological analysis. For a subset of tumors immunohistochemical staining was performed with the antibodies B220 (Becton Dickinson), Ig kappa (Southern Biotechnology), and F480 (Serotec) on formalin-fixed, paraffin-embedded tissue. B220 and Ig kappa staining were performed with a citrate buffer unmasking step. F480 staining was performed with a trypsin unmasking step, and for F480 staining, all incubations and washes were done in 0.05% Triton X 100.

Southern Blotting

Tumor tissue was digested with proteinase K overnight, and DNA was isolated following phenol chloroform extraction. Following EcoR1 digest, DNA was loaded on a 0.8%

agarose gel and transferred to a nylon membrane N+ (Amersham). Membrane was hybridized to a P32 labeled probe of a 1.9Kb BamH1-EcoR1 J_H genomic fragment including mouse J_H3 and J_H4 sequences for detection of gene rearrangements.

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Chapter 5

Overview and Future Directions

A. Cell context-specific effects of p53 S23A mutation

p53 undergoes extensive posttranslational modification in response to stress, yet the in vivo relevance of most of these modifications has not been determined. Human Ser20 has been implicated in p53 stabilization in vitro. Therefore, we targeted the murine equivalent of this site, converting Ser23 to alanine, prohibiting its phosphorylation under situations in which p53 was regulated under its endogenous promoter, to determine the effects of this mutation in vivo (MacPherson et al., 2004a).

Since the phosphorylation of this residue was implicated in conferring p53 stabilization, we examined p53 protein stability in various cellular contexts. In MEFs we found little difference between mutants and controls in levels of p53 stabilized following IR, indicating that phosphorylation of this residue is not absolutely required for p53 stabilization. We also found that p53 target genes were induced and cell cycle arrest function was intact. In thymocytes irradiated in vitro, we found a clear decrease in p53 levels with Ser23 mutation, and were able to show that p53 half-life following irradiation was reduced with Ser23 mutation, consistent with a role for this residue in conferring p53 protein stability following DNA damage. However, while reduced thymocyte p53 stabilization was observed in cultured thymocytes, we found a much stronger effect of Ser23 mutation when the animal was irradiated in vivo and p53 levels in the thymus were examined in the absence of any cell culture stress. Also, in the developing cerebellum irradiated in vivo, there was a particularly striking reduction in the levels of p53 stabilized with Ser23 mutation. In some cellular contexts, such as under cell culture stress, p53 may be sensitized to allow for stabilization in the absence of Ser23

phosphorylation, while in other contexts, phosphorylation of this residue may be critical. Both cell culture conditions as well as cell type differences may play a role and it would be interesting to explore the reason behind differences in effects of Ser23 mutation. For example, the factors that regulate p53 Ser23 phosphorylation or other regulators of p53 stability may be expressed differently in different tissues, or *in vivo* vs. under cell culture conditions. Also, other Ser23 independent pathways involved in p53 stabilization may be more active under different conditions.

Along with decreased p53 stabilization we found decreased apoptosis with Ser23 mutation. This was shown in thymocytes, in the developing nervous system and in splenic B-lymphocytes following irradiation *in vivo*. Importantly, we did not find a complete resistance to apoptosis which has been reported in thymocytes and in the developing nervous system of *p53*-/- animals following IR (Herzog et al., 1998; Lowe et al., 1993). The resistance to apoptosis observed can be explained by the decrease in p53 protein stability, however it remains possible that other activities of p53 may be compromised.

This is the first demonstration of a p53 phosphorylation site mutant prone to spontaneous tumorigenesis, strongly supporting the hypothesis that phosphorylation of this residue is important for p53 biology. While *p53*-/- animals rapidly succumb to thymic T-lymphomas, homozygous Ser23Ala mice largely developed B-lineage tumors. The tumor spectrum was interesting given results from the Lozano lab in which gene targeting of a different point mutated p53 allele (Arg172Pro) led to a complete lack of p53-dependent

apoptosis but partial cell cycle arrest function in various cellular contexts. This Arg172Pro mutation was associated with a switch in tumor spectrum from early, aneuploid tumors in *p53*-/- mice to later arising diploid lymphomas (including B cell tumors) and sarcomas. Cell cycle arrest and maintenence of genomic stability were implicated as being important for the p53-mediated suppression of T-lymphomas. The Arg172Pro allele led to a complete loss of apoptotic function while the Ser23Ala mice had a strong, but incomplete impairment of apoptotic function in response to IR. One possibility is that these point mutant alleles represent an allelic series with the Arg172Pro allele representing a severe hypomorph, while Ser23Ala mutation representing a less severe allele. This would be consistent with the kinetics of survival of the mutant mice and the extent of impairment in p53 apoptotic function observed (Liu et al., 2004; MacPherson et al., 2004a). Overall, the data are consistent with a threshold model of p53 function where different levels of p53 are needed for different effects, i.e. low levels of p53 function can confer cell cycle arrest and can suppress thymic T-lymphomas, while high levels are needed to suppress B-lymphomas. Alternatively, specific pathways may feed through to affect Ser23 phosphorylation in different contexts, for example the Ser23 phosphorylation pathway may be more active in some cell types than others, and this remains to be investigated.

The nature of the p53 activity that plays a role in suppressing B-cell lineage tumors is not known. We tested the response of splenic B cells to irradiation and found partial resistance with Ser23 mutation. However, we do not know if this is relevant to B-lineage tumorigenesis, i.e. the p53 inducing signals important for suppression of these tumors

may be quite different from the DNA damage induced by ionizing irradiation. Thus, a better understanding of the genesis of these tumors is needed to understand the possible role of p53 in the suppression of these tumors. Further molecular characterization of these tumors may provide clues as to how p53 may be involved.

A limitation of our study was that phosphorylation of Ser23 residue in the mouse could not be shown due to the poor quality of available antibodies. Thus, it is conceivable that effects could be due to other consequences of having an alanine at Ser23 other than the absence of phosphorylation. It will be important for future studies to determine if this residue is phosphorylated in the mouse, perhaps through use of an approach such as mass spectrometry. This approach would also be useful for determining if change in the phosphorylation of other residues occurs with Ser23A mutation. For example, phosphorylation of one site may be required for phosphorylation of other sites. This idea that phosphorylation site interdependence may play a role in the phenotype is supported by studies using ectopic expression of p53 phosphorylation site mutants in which Ser20 phosphorylation was required for Thr18 phosphorylation (Saito et al., 2003). Some of the observed phenotypic effects may be due to other modification events that rely on Ser20 phosphorylation.

B. pRB in development and retinoblastoma suppression

Cell-autonomous vs. non-cell-autonomous effects of *Rb* mutation

This work was rooted in efforts to understand the nature of the signals that led to p53 dependent apoptosis in the *Rb*-/- CNS. Examination of candidate mediators of p53

activation such as Arf did not lead to evidence of any involvement in p53-mediated apoptosis (Tsai et al., 2002). Hypoxia is a physiological activator of p53, and in tumors it has been shown that functional inactivation of the Rb family sensitizes tumor cells to hypoxia-mediated p53-dependent apoptosis (Graeber et al., 1996). I hypothesized that hypoxia due to erythropoietic defects in *Rb*-/- embryos contributed to the CNS apoptosis and found evidence of hypoxia induced gene expression in the *Rb*-/- CNS (MacPherson et al., 2003). Our finding that some of the apoptosis in *Rb*-/- embryos is due to non-cell-autonomous effects of a hypoxic environment was important as it helped in the interpretation of many compound mutant studies that had posited more direct effects of pRB loss on the observed phenotypes (Guo et al., 2001; Macleod et al., 1996; Simpson et al., 2001; Tsai et al., 1998; Tsai et al., 2002; Ziebold et al., 2001). For example, where E2F1 and E2F3 were proposed to signal to p53 in a relatively direct pathway, these experiments showed that needed cofactors in apoptosis resided outside of the brain. Our work was complemented by work of the Leone lab who found that rescue of placental defects in *Rb*-/- mice led to survival of *Rb*-deficient animals to birth, with phenotypes of the rescued animals similar to those we described (de Bruin et al., 2003; Wu et al., 2003). The phenotypes uncovered in the *Rb*-/- placenta, involving expansion of trophoblast cells in the placental labyrinth layer and decreased placental transport is consistent with the idea that oxygen transport to the embryo may be impaired (Wu et al., 2003). In addition, the placental defects also likely affected embryonic fetal liver erythropoiesis (de Bruin et al., 2003). It is also possible that other effects of placental insufficiency in addition to hypoxia could contribute to the *Rb*-/- phenotypes.

In other tissues, apoptosis was not dependent on hypoxia downstream of pRB loss such as the lens, ganglia of the peripheral nervous system (PNS) and the retina (MacPherson et al., 2003; MacPherson et al., 2004b). This is also important as apoptosis in the PNS was previously shown to be p53-independent (Macleod et al., 1996) and Rb-deficient retina is also p53-independent (MacPherson et al., 2004b). It is possible that for development of certain tumors, such apoptotic pathways may have to be evaded through mutation. Thus, it will be important to understand how p53-independent apoptosis is mediated in the absence of pRB in vivo.

An additional advantage of the *Rb* conditional deletion strategy used is that it allowed us to uncover effects of *Rb* deletion at later stages than could previously have been examined. For example, *Rb* loss led to increases in brain size (MacPherson et al., 2003) as well as regional brain defects at birth (MacPherson and Jacks, unpublished). Histological defects in the *Rb*-deficient retina were observed, and we focused on the role of pRB in the biology of the retina.

The *Rb*-deficient retina

Since the cloning of the *RB* gene as being responsible for retinoblastoma nearly 20 years ago, little had been learned about *RB* function in the retina, or of the basis of the sensitivity of this tissue to tumorigenesis upon *RB* deletion. In *Rb*-/-:+/+ chimeric animals, retinoblastomas were also never found (Maandag et al., 1994; Williams et al., 1994). However, apoptosis was observed, indicating that pRB plays a role in the development of the murine retina. This thesis describes findings that homozygous *Rb*

loss in the retina did not lead to retinoblastoma in the mouse, but did lead to dramatic effects on retinal development. pRB loss led to proliferation defects, especially in the differentiating compartment of the retina, and led to an extension of retinogenesis. This was balanced by apoptosis; in late stages, rod bipolar cells, some rod photoreceptors and retinal ganglion cells were depleted, and adult retinas lacking pRB were hypocellular. pRB loss appears to cause cell cycle exit defects, however all cells eventually either exited the cycle or underwent apoptosis and thus, retinoblastomas never arose. With dual inactivation of *Rb* and *p130*, however, mice were highly retinoblastoma prone.

Others have also recently performed analyses of pRB function in the retina. The Bremner lab took a similar approach, using the same *Pax6 alphaCre* transgenic to drive *Rb* deletion in the retina, and obtained very similar results (Chen et al., 2004). Also, by labeling ectopically proliferating cells with BrdU and markers of specified cells, they concluded that in the absence of pRB, ectopic division occurs in all cell type specified cells (cells that they indicate should normally be postmitotic). This group concluded that progenitors are not affected by pRB loss. However, some of the markers of specified cell types this group used (e.g. Chx10 to mark bipolar cells) are also expressed more broadly in progenitors at earlier stages (Liu et al., 1994). Thus, the question of whether pRB loss affects progenitors or specified cells or both was not resolved. Similar to our findings, this group also found that there was differential survival depending on the cell type, as some rods, as well as bipolar and ganglion cells were depleted in the absence of pRB (Chen et al., 2004; MacPherson et al., 2004b). This may have relevance for retinoblastoma in that the cell of origin of retinoblastoma may have some intrinsic

resistance to apoptosis upon *Rb* deletion. Whether this death resistant cell is a progenitor cell, or a cell-type specified cell that would otherwise be postmitotic is not clear.

Another group took different approaches and reported that pRB loss leads to increased progenitor proliferation as well as defects in rod photoreceptor development (Zhang et al., 2004a). Retinal explant cultures have been well described to mimic much of normal retinal development and have been useful in the study of retinal development in mice that undergo developmental lethality. In explant cultures derived from germline *Rb* mutant E13.5 retinas, cultured for 12 days, this group found substantially more cells in *Rb* mutant retinas. They also found that BrdU incorporation was increased in such explants. In contrast to our results, they did not find increased apoptosis in their system, and the only cell-type specific effects involved rod photoreceptors, which exhibited decreased numbers and altered nuclear morphology with pRB loss. The lack of apoptosis and increase in overall retinal cell numbers is different from the high apoptosis and cell loss we, and the Bremner group, observed in vivo. This suggests that the effects of pRB loss in the explant system is different than in vivo. For example, survival factors in the media could inhibit the death of Rb-/- cells. The idea of an in vivo vs. in vitro difference is consistent with some of the data found in Zhang et al. By injecting virally expressed Cre into the retina of newborns, the effects of pRB loss on emergence of bipolar cells in clones could be examined in vivo (see Supplemental Table 2 of (Zhang et al., 2004a)). Interestingly, while bipolar cells (a postnatally generated cell type) emerged in controls at expected numbers, they were completely absent from clones with either acute *Rb* deletion or with E1A addition. These data argue for a cell autonomous role for pRB in bipolar

cells development in vivo. If survival factors can indeed allow *Rb*-/- bipolar cells to survive in culture, it would be interesting to try to dissect the signals that allow these *Rb*-/- cells to survive. This group also took another in vivo approach, using *Cre* driven by the *Chx10* promoter to delete *Rb* in vivo, confirming their findings that Rb loss causes rod defects, but they did not report phenotypes in other *Rb*-/- cell types.

Inheritable models of retinoblastoma

It was surprising that when *Rb*+/- mice were generated, such mice never developed retinoblastomas (Jacks et al., 1992). Chimeric animals with cells lacking both pRB and p107, however, were retinoblastoma prone, showing that retinoblastomas can develop in the mouse (Robanus-Maandag et al., 1998). The chimera experiments suggested that either functional redundancy or active compensation involving p107 may prohibit retinoblastoma formation in mice lacking pRB. The chimeric system was not ideal as a tool for understanding retinoblastoma development and generating tumor material, in part due to poor survival of the mice to adulthood. This thesis describes the generation and initial use of breedable models of retinoblastoma. We found that either p130 or p107 can act as suppressors of retinoblastoma in *Rb*-/- retinas. Chimeric animals with cells lacking pRB and p130 were also recently described and these animals also developed histologically similar retinoblastoma (Dannenberg et al., 2004). Our model, and the inheritable models of retinoblastoma that others have generated (Chen et al., 2004; Zhang et al., 2004b) will be valuable for the field in testing of new therapies for retinoblastoma, as well as for understanding the genetic alterations needed for retinoblastoma, and the retinoblastoma cell of origin. These retinoblastoma models result in tumors that exhibit

amacrine cell characteristics, staining for amacrine markers such as syntaxin and calretinin. The cell of origin of the tumors, and the similarities to human retinoblastomas are important, unresolved questions. Chen et al (2004) proposed that murine retinoblastomas arise from a death-resistant amacrine cell. It is also possible that the tumors may arise from an earlier progenitor cell type that preferentially expresses amacrine characteristics. Emerging evidence indicates that there is heterogeneity in marker expression in murine retinoblastomas; some amacrine cell markers are expressed in a focal fashion in murine retinoblastomas, also, cone arrestin was found in murine retinoblastomas lacking *Rb, p107* and *p53* (Zhang et al, 2004) and we find that retinoblastomas lacking *Rb* and *p130* express the photoreceptor marker S-antigen (MacPherson and Jacks, unpublished). A more complete analysis and comparison to human retinoblastoma is needed, perhaps at a genomic level using expression profiling, to determine the similarities and differences between the human and murine tumors.

Some critical questions concern the role of apoptosis and the role of p53 in retinoblastoma development. Early experiments using IRBP-promoter driven E7 expression to inactivate the pRB family in the differentiating photoreceptor compartment resulted in apoptosis, however retinal photoreceptor tumors arose with p53 inactivation (Howes et al., 1994). While this suggests that p53 may suppress retinoblastoma development, the tumors that arise with pRB and either p107 or p130 loss exhibited very different histological and cell-type characteristics, and emerge from the inner nuclear layer (Chen et al., 2004; Dannenberg et al., 2004; MacPherson et al., 2004b; Robanus-Maandag et al., 1998). Thus, the origins of these retinal tumors may be very different,

and the role of p53 in retinoblastomas that arise with pRB family member loss is not clear. Therefore, we tried to determine if p53 would cooperate with pRB loss to cause retinoblastomas, and found that retinoblastomas did not result with *Rb* and *p53* mutation, and we found no rescue of apoptosis in the absence of p53 (MacPherson et al., 2004b). Interestingly, Zhang et al compared conditional *Rb/p107* vs. triple *Rb/p53/p107* mutants, in which *Rb* deletion was controlled by *Chx10* promoter driven-*Cre* in a sebset of retinal progenitors and both of these groups of mice develop retinoblastomas. However, the absence of p53 contributed to aggressive tumors that exhibited an increased frequency of invasion into the anterior chamber of the eye. Together, these data support the idea that p53 loss may contribute to the progression of retinoblastoma, but does not act as a switch that cooperates with pRB loss to allow a tumor to initially form. Interestingly, the one reported case of a *p53* mutation in human retinoblastoma was from a retinoblastoma metastasis (Kato et al., 1996), with no mutations in any of 26 primary retinoblastomas (Kato et al., 1996), or in other independent analysis of *p53* status in retinoblastoma (Gallie et al., 1999).

Compensation in pRB-deficient retinas

The ability of cells to adjust to perturbations induced by genetic manipulation is remarkable and can reflect plasticity in development or the existence of feedback responses that sense signaling pathway alterations and respond to minimize deleterious effects. For example, the Sicinski group has shown that the three D-type cyclins exhibit tissue specific expression patterns. With deletion of two of the three D-type cyclins, compensation involving upregulation of the levels of the remaining family member often

occurred in a tissue in which that cyclin would never normally have been expressed, and effects of the perturbations were thus minimized (Ciemerych et al., 2002). Similarly, in many cell culture systems, loss of pRB leads to a substantial increase in p107 levels (Hurford et al., 1997; Ruiz et al., 2004; Sage et al., 2003; Schneider et al., 1994). Because loss of pRB and p107 in the mouse can lead to retinoblastoma, the idea that *Rb*-/- murine retinas do not develop retinoblastomas is due to compensation involving p107 is attractive. Surprisingly, we found that p107 levels were not increased in *Rb*-/- embryo retinas at E18.5 (MacPherson et al., 2004b). In contrast, in a microarray experiment profiling retina RNA extracted from *Rb*-/- retinal explant cultures, p107 was found upregulated, along with many cell cycle regulated genes (Zhang et al., 2004a). However, as p107 is a gene induced in cycling cells as they proceed to S-phase, any perturbation that increases the proportion of cycling cells in a population would be expected to increase p107 levels regardless of compensation. An increase in the proportion of cycling cells is consistent with increased cell numbers upon Rb loss in the explant system studied, as well as with other data that show an increase in overall BrdU incorporation at postnatal stages in the *Rb*-deficient retina (Chen et al., 2004; MacPherson et al., 2004b). Instead of overall increases in p107 (or p130) levels, we found that p107 phosphorylation shifted with Rb loss, suggesting an increase in activity, and this was associated with decreased cyclin D1 levels. One unique feature of the developing retina is that cyclin D1 is expressed at extremely high levels that are not found in any other developing tissue (Sicinski et al., 1995). High cyclin D1 levels may allow for rapid progenitor expansion early in retinal development, but this may have adverse consequences if *Rb* is inactivated; the high levels of cyclin D1 may inactivate the pocket protein family, causing a

sensitization of this tissue to cancer with pRB loss. In other tissues, active p107 or p130 may be able to minimize the effects of pRB loss. Thus, the possibility that modulation of cyclin D1 levels may occur in response to pRB loss is interesting, and it would be interesting to know if this also occurs in human retinal cells. Also, while we have observed this response in bulk retinal extracts, it is not known if this, or other mechanisms of compensation act in the cell of origin of retinoblastoma. In a cell culture system, ectopic expression of any of the pocket proteins could increase *cyclin D1* promoter activity, and in one study this was mediated my NfkappaB activation (Takebayashi et al., 2003). We have not as yet determined whether the NFkabbaB pathway is perturbed in the *Rb*-deficient retina. If cyclin D1 downregulation were prevented, for example through forced expression of cyclin D1 another critical question is whether cooperation with pRB loss in retinoblastoma development will occur.

p107 and p130 as tumor suppressors

The suppression of retinoblastoma by p107 or p130 in the context of pRB deficiency raises the possibility that multiple members of this family may have to be inactivated to inactivate the pathway. Consistent with this idea, DNA tumor viral proteins that inactivate pRB, such as E7, Large-T and E1A also have evolved to inactivate each of the pocket protein family members. Interestingly, the pRB pathway is much more frequently inactivated by deregulation of upstream pRB regulators vs. inactivation of *RB* itself. Tumorigenic effects of *Cyclin D* or *CDK4* amplification or overexpression, or *p16INK4A* loss is often attributed to inactivation of pRB, however, these events likely impinge on the other RB family members, and inhibition of p107 and p130 may indeed be critical for

some of the downstream tumorigenic effects. These results also raise the possibility that perhaps *p130* and/or *p107* are mutated in human cancers that also mutate *RB*. It is interesting to note that *p130* is in a region of chromosomal loss in human retinoblastoma, that includes the long arm of chr. 16. We have hypothesized that selection for *p130* inactivation may be driving chromosome 16 deletion, and thus, we have started sequencing the human *p130* locus from 48 human retinoblastoma samples obtained from Ted Dryja's lab. It is also possible that *p130* could be inactivated in through other means, such as via promoter hypermethylation.

Current and future directions

Critical questions that result from this work concern (1) the cell of origin of retinoblastoma, (2) the genetic changes in addition to *RB/Rb* loss required for retinoblastoma, and (3) the nature of signaling pathways controlled by pRB in the retina. My current and future work will focus on these questions.

Regarding the cell of origin of retinoblastomas in the mouse model, preliminary experiments indicate that the tumors arise in a very specific location at the extreme periphery of the retina (MacPherson and Jacks, unpublished). This is also consistent with a recent report in which chimeric animals with *Rb* and *p130* inactivation were generated and retinoblastomas also arose from the retina periphery (Dannenberg et al., 2004). This suggests that there is a specific niche of cells sensitive to tumorigenesis with *Rb* and *p130* mutation and it will be important to determine the properties of the relevant cells. Interestingly, an analogous location called the ciliary marginal zone in fish and

amphibians (organisms which undergo retinogenesis in adulthood) has been characterized as a niche for adult stem cells (reviewed in (Hitchcock et al., 2004)). Also, in a mouse hedgehog pathway mutant (*Ptch*+/- mice), persistent progenitor cell proliferation was found at the extreme periphery of the retina in adulthood (Moshiri and Reh, 2004). It will be critical to evaluate the proliferating cells for progenitor and mature cell markers to help determine the characteristics of cells in the earliest pre-tumorigenic lesions in the *Rb/p130* retinoblastoma model.

α-Cre Rblox/lox;p107-/- animals often develop unilateral retinoblastoma, and tumor penetrance is incomplete ((Chen et al., 2004)MacPherson and Jacks, unpublished). This is consistent with previous data from *Rb/p107* deficient chimeras (Robanus-Maandag et al., 1998) and suggests that *Rb* and *p107* inactivation is not sufficient for retinoblastoma development, and other genetic changes may be needed. In humans, there is evidence based on comparative genomic hybridization analyses that specific genomic regions consistently undergo chromosomal gains or loss, arguing for additional mutations in retinoblastoma (Mairal et al., 2000). The most frequent regions of changes involve chromosome 6p and 1q (each amplified in over 50% of retinoblastomas) and loss of 16q in over 30% or retinoblastomas. To determine what other specific gene changes in addition to *Rb* loss may be occurring, it will be interesting to perform comparative genomic hybridization analyses on murine tumor material to assess regions of chromosomal gain or loss. By comparing the genes in regions of change to similar data from human samples, a large list of candidate genes may be narrowed down. Once candidate genes are identified, such genes could be assayed for cooperativity with *Rb* loss

in vivo in the retina using conditional mutation or viral based retinal manipulation. This will help to address the question of the nature of additional signals in addition to *Rb* loss needed for tumorigenesis.

Finally, it will also be interesting to use genetically defined retinas to uncover signaling pathways altered with *Rb* deletion, or with *Rb* and *p107* or *p130* loss to gain a better understanding of the signaling changes that mediate the phenotypes observed downstream of pRB in the retina in vivo. While much work has been done on pathways downstream of pRB, most of these studies have been performed on tissue culture cells and not directly on tissues in which strong phenotypic effects are observed with Rb deletion in vivo. Microarray experiments followed by functional validation of candidate genes will be done. A focused analysis of the role of pRB in the retina in vivo may help to uncover the reasons for the exquisite sensitivity of this tissue with pRB loss, as well as general roles for pRb in vivo.

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Appendix 1

ARF mutation accelerates pituitary tumor development in *Rb*+/- mice

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The author performed all of the Southern Blots as well as the laser-capture loss-of-heterozygosity analyses in early lesions. The author also performed many of the necropsies as well as the western blot for ARF expression in pituitary tumors. K.T. performed the remainder of this work and initiated these studies. First Authorship was shared between KT and DM.

ABSTRACT

Mice heterozygous for the *Rb* tumor suppressor gene develop pituitary and thyroid tumors with high penetrance. We demonstrate here that loss of the *ARF* tumor suppressor strongly accelerates intermediate lobe pituitary tumorigenesis in *Rb* heterozygous mice. These effects in the pituitary are greater than those conferred by *p53* loss in that *Rb+/-; ARF-/-* mice display significantly more early atypical lesions than *Rb+/-; p53-/-* mice. Also, *Rb+/-; ARF-/-* compound mutants do not develop many of the novel tumors or pre-cancerous lesions seen in *Rb+/-; p53-/-* compound mutants. Although complete loss of *ARF* expression is not obligatory for pituitary tumorigenesis in *Rb+/-* mice, alterations of the *ARF* locus are observed in tumors from *Rb+/-; ARF+/-* mice, consistent with a selective advantage of *ARF* inactivation in this context. We conclude that inactivation of *ARF* acts more broadly than that of *p53* in connecting abrogation of the *Rb* pathway to tumorigenesis.

INTRODUCTION

The retinoblastoma (*RB*) tumor suppressor gene is critical for control of the G1-S cell cycle transition and tumor suppression (1). Individuals heterozygous for an *RB* mutation are predisposed to retinoblastoma and osteosarcoma, and *RB* is also inactivated in a wide variety of spontaneously-arising human cancers (2). In addition, the protein product, pRB, is regulated by a number of factors that are also mutated or otherwise inactivated in other familial cancer syndromes and in sporadic tumors (2). These include amplification and overexpression of cyclin D1 (*CCND1*) in carcinomas, amplification and dominant activating mutation of *CDK4* in melanomas, and mutation or deletion of *p16INK4A* (*CDKN2A*) in multiple tumor types (3-5). Because this pathway appears to be a central regulator of tumor suppression, elucidation of the downstream consequences of its inactivation is critical for understanding the molecular bases of cellular transformation and tumor development.

pRB interacts with a number of cellular proteins to regulate a multitude of cellular events. One such role is in the regulation of the late G1 to S transition of the cell cycle through its interactions with the E2F family of transcription factors. In addition, pRB is known to regulate other cellular functions including differentiation through other cellular factors, and is known to be important in the development of multiple cell lineages (6, 7).

The effects of *Rb* mutation have been studied extensively in mice (8-10). Homozygous *Rb* mutant (*Rb*-/-) embryos die in mid-gestation due to a defect in hematopoiesis. *Rb* heterozygous mice (*Rb*+/-) develop pituitary tumors and thyroid tumors with high penetrance as well as a number of neuroendocrine tumors (9, 11-13), representing a syndrome of multiple endocrine

neoplasia (13). These tumors exhibit loss of the wild-type allele of *Rb* consistent with a requirement for this event in tumorigenesis (11, 12, 14). By generating mice mutant for combinations of genes, we and others have been able to use this system to probe downstream components of the *Rb* pathway and establish genetic interactions that impinge upon the consequences of *Rb* inactivation and subsequent tumor formation (15-18).

The analysis of chimeras composed partly of *Rb* homozygous mutant ES cells showed that inactivation of both alleles of *Rb* is a required, rate-limiting step for pituitary tumorigenesis (19, 20). Pituitary-specific ablation of homozygous conditional alleles of *Rb* by an rPOMC promoter-driven Flp enzyme resulted in mice with significantly shorter tumor latency than that of $Rb^{frt/-}$ controls lacking the enzyme or *Rb*+/- mice (21), confirming this result. Other genes, notably upstream regulators of pRB, also contribute to pituitary tumorigenesis in the mouse and further highlight the centrality of the *Rb* pathway in this process. Mutation of members of the *Ink4* and *Cip/Kip* families of cyclin-dependent kinase inhibitors (CKIs) predispose to pituitary hyperplasia. Mice lacking *p27Kip1* (*Cdkn1b*) or *p18Ink4c* (*Cdkn2c*) develop pituitary hyperplasia, and the compound mutants for *p21Cip1* (*Cdkn1a*), demonstrate enhanced tumorigenesis (22-25).

A number of tumor models based on selective inactivation of *Rb* family function have been developed as well, including strains that express a truncated SV40 large T (TgT121) antigen in the choroid plexus (26) or human papillomavirus (HPV-16) E7 in photoreceptors (27) or ocular lens (28). These strains have been used to examine the genetic interactions between inactivation of *Rb* and other tumor suppressor genes in tumorigenesis.

By crossing the TgT121 strain into the *p53* null background, it was shown that the proliferative advantage of tumor cells achieved through abrogation of *Rb* family function resulted in high levels of *p53*-dependent apoptosis contributing to the long tumor latency. Removal of this latter mechanism of tumor suppression resulted in rapid growth of tumors (27, 29, 30). Subsequently, the study of *Rb*+/-; *p53*-/- germline mutant mice revealed clear cooperative roles for loss of both tumor suppressor genes in the development of pinealoblastoma, bronchial epithelial hyperplasia, and pancreatic islet cell hyperplasia, all of which were lesions not found in high frequency in *Rb*+/- or *p53*-/- mice (15, 16), although the predisposition to bronchial and islet cell hyperplasias differs for mice harboring a different targeted *Rb* allele (13). Furthermore, the pinealoblastomas exhibited LOH for both *Rb* and *p53* in *Rb*+/-; *p53*+/- mice indicating that the inactivation of both genes are obligate genetic events in the development of these lesions (15). Surprisingly, pituitary tumorigenesis in *Rb*+/- mice was not significantly affected by inactivation of *p53*, and LOH at this locus was observed very infrequently (1/16) in pituitary tumors isolated from *Rb*+/-; *p53*+/- mice (15).

A previously unexplored candidate interactor is the *ARF* tumor suppressor, which like the CKIs has growth-suppressive properties, but is not known to directly affect CDK activity or components (31). *ARF* was identified as an alternative transcript of the *Ink4a* locus possessing a unique first exon (1b) and promoter (31). *ARF* functions as a tumor suppressor gene in mice (32, 33) and there is evidence to suggest such a role in humans as well (34-36). Biochemically, ARF inhibits multiple functions of MDM2 leading to stabilization of p53 (37-42). *ARF* has been shown genetically to be required for efficient *p53*-dependent responses to cellular stresses

including overexpression of oncogenes such as *RAS*, *MYC*, and *E2F-1* as well as DNA damage (43-47). Furthermore, ARF is upregulated in *Rb*-deficient cells (47) and has been shown to be a transcriptional target of E2F-1 (44, 48, 49). Therefore, as a potential bridge between the *Rb* and *p53* pathways, *ARF* would be expected to play a prominent role in a number of processes including tumorigenesis. In at least one strain of *Rb*+/- mice, the development of pinealoblastoma, bronchial epithelial hyperplasia, and pancreatic islet cell hyperplasia occurred in appreciable frequency only in *Rb*+/-; *p53*-/- mice (15). Given the role of ARF in activating p53 in a variety of contexts, one might expect the inactivation of *ARF* to have similar effects on the *Rb*+/- background.

ARF also has functions that are not dependent on *p53*. For example, *ARF*-/- mice have eye abnormalities associated with failed hyaloid vascular system regression that is not dependent on *p53* (50). Also, overexpression of *ARF* has been demonstrated to impair S-phase progression in *p53*-deficient tumor cell lines (51). It is not clear however if *p53*-independent functions of *ARF* contribute to tumor suppression. The lack of a clear cooperative effect between loss of *p53* and loss of *Rb* in promoting intermediate lobe pituitary tumorigenesis (15, 16) raises the interesting question of whether loss of *ARF* can affect this process. Such an interaction might represent effects much broader than those conferred by *p53* inactivation, perhaps demonstrating roles for *ARF* in tumorigenesis beyond that of a simple link between *Rb* inactivation and *p53*.

MATERIALS and METHODS

Mice. *Rb+/-; ARF-/-* animals were generated by breeding *Rb+/-* mice to *ARF-/-* mice on a mixed 129 x B6 background to generate compound mutants. *Rb+/-; ARF-/-* and *Rb+/-; ARF+/-*, and *ARF-/-* mice were intercrossed to generate the *Rb+/-; ARF-/-* compound mutant mice. A similar strategy was used to generate mixed 129 x B6 *Rb+/-; p53-/-* compound mutant controls. Survival curves were compiled from mice that were sacrificed when moribund. The logrank (Mantel-Haenszel) test was performed to establish statistical significance (Prism 3, GraphPad Software).

Tumor and early lesion analysis. For tumor analysis, mice were sacrificed, heads were cut along the midline and fixed in 10% formalin overnight, processed, mid-sagittal sections cut and stained for BrdU and TUNEL (see below). For EAPs, mice were anesthetized and perfused with 4% paraformaldehyde (14) or pituitaries microdissected immediately following sacrifice, fixed in Bouin's fixative at room temperature for 16 hours and rinsed in 70% ethanol. Tissues were processed and paraffin serial sections cut at 4 mm. Sections were stained with hematoxylin (Mayer's) and eosin and screened at 600X to 1000X for early lesions. The proliferation index was calculated by counting the percentage of BrdU-positive nuclei out of fields containing approximately 500 nuclei, and significance tested using a paired t-test. Volumetric analysis of EAPs was conducted by measuring the diameters of each lesion at the widest point in each dimension, assuming an ellipsoid shape, and calculating the volume as ($\pi/6$ * d1 * d2 * d3).

TUNEL and Immunochemistry. Apoptosis was assayed using the TUNEL assay (52). For BrdU analysis, a mixture of BrdU (5-bromo-2'-deoxyuridine; Sigma) and FdU (5-fluoro-2'-deoxyuridine; Sigma) was injected intraperitoneally (100 mg and 10 mg / gm body weight, respectively) one hour prior to sacrifice. Sections were rehydrated, blocked in 3% H_2O_2, processed in pepsin and HCl and incubated with a mouse monoclonal anti-BrdU antibody

(Becton Dickinson). All immunohistochemistry employed the ABC peroxidase detection system (Vector Laboratories).

ARF expression. Whole pituitary tumor samples were lysed in 100mM Tris pH8, 100mM NaCl, 1% NP40, with Complete protease inhibitor tablets (Roche). 450 μg of total protein was electrophoretically separated on 12.5% PAGE, blotted to PVDF (Millipore) membrane and probed with anti-ARF antibodies (Novus, 1:2000).

Southern and northern blot analysis. Pituitary tumors were microdissected immediately following sacrifice and frozen in liquid nitrogen. Southern blot was performed using standard protocols with DNA digested with Pst I / Asp 718i for *Rb* southern or AflII for the *ARF* southern. Blots were hybridized with radiolabelled probes spanning exon 3 of the *Rb* locus and exon 1b of the *ARF* locus. Northern blot analysis was performed using standard protocols with 10μg total RNA isolated from pituitary tumors. Probe was made to exon 1b of the *ARF* locus or to *GAPDH* cDNA.

Laser capture microdissection - PCR. Samples processed for laser capture analysis were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned and stained with hematoxylin and eosin. Early abnormal proliferates or surrounding normal pituitary tissue was captured using a PixCell II laser capture microdissection system (Arcturus). LCM-captured samples were digested in proteinase K overnight at 42°C, inactivated for 10 minutes at 95°C, and used in 50μL (2mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH8.3), dNTPs 200μM, primers 0.4μM (RI3, RbpA) or 0.8μM (Rbint3f), 0.001% gelatin, 1 μL Taq Polymerase (Amplitaq Gold)) PCR reactions (94°C for 10 min, 40 x (94°C for 30 sec, 61°C for 1 min, and 72°C for 1 min), 72 ° C for 10 min). Primers: Rbint3f (common) 5'-CACCATGTGCAATGCTTGA-3', RI3 (wild-type) 5' CCCATGTTCGGTCCCTAG-3', RbpA (mutant), 5'-ACGAGATCAGCAGCCTCTGT-3'. PCR

products were resolved on 2% agarose gels, with approximately 130 bp wild-type band and 160 bp mutant bands.

RESULTS

Loss of ARF dramatically accelerates pituitary tumorigenesis in Rb+/- mice

A comparison of the survival curves of *Rb*+/- mice versus *Rb+/-; ARF-/-* compound mutant mice on a mixed 129 x B6 background revealed a strong interaction between the inactivation of these two tumor suppressor genes. Whereas *Rb*+/- mice (n=37) in this cohort survived for 276 +/- 41 days (median = 277 days), the *Rb+/-; ARF-/-* mice (n=32) survived for of 168 +/- 49 days (median = 168.5 days; $p<0.0001$) (Fig. 1). Clinical observation and necropsy of these mice revealed that virtually all of these mice died of intermediate lobe pituitary tumors. While *ARF-/-* mice have not been observed to have pituitary or thyroid lesions ((33) and data not shown), *Rb+/-; ARF-/-* mice were highly predisposed to development of pituitary tumors as well as thyroid C cell carcinomas.

Importantly, *Rb+/-; ARF-/-* mice did not exhibit the pancreatic islet cell hyperplasia (0/10), pinealoblastoma (0/14), or bronchial epithelial hyperplasia with appreciable frequency (0/10), as previously reported in *Rb+/-; p53-/-* mice ((15, 16), data not shown). Therefore, in these tissues, *ARF* loss is not equivalent to *p53* loss, implying that p53 is regulated by other means. Similar findings have been demonstrated in a mouse model for medulloblastoma in which loss of *p53* but not *ARF* accelerates tumor development in animals heterozygous for Patched (*Ptch*). (53).

Pituitary tumors of Rb+/-; ARF-/- mice have higher proliferative capacity

Examination of tumors isolated from *Rb+/-; ARF-/-* mice and *Rb*+/- controls revealed that those derived from the compound mutants developed much faster even though end-stage tumors from both populations were histologically indistinguishable. Tumors in 3-5 month-old *Rb+/-; ARF-/-*

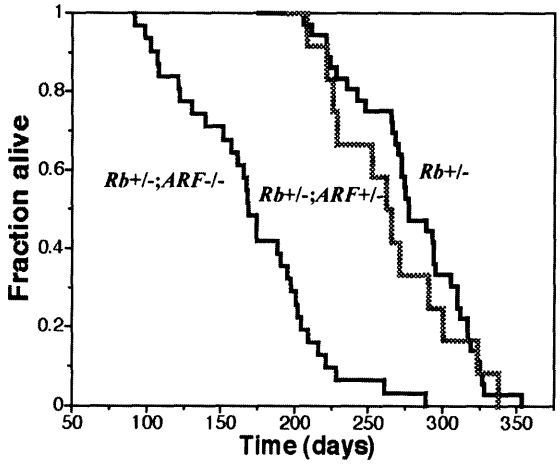

Fraction alive
1
0.8
0.6
0.4
0.2
0
50
100
150
200
250
300
350
Time (days)
Rb+/-;ARF-/-
Rb+/-;ARF+/-
Rb+/-

Figure 1. *Rb*+/-; *ARF*-/- compound mutant mice exhibit significantly decreased survival relative to *Rb*+/- and *Rb*+/-; *ARF*+/- mice.

Survival curves of *Rb*+/- (n=37), *Rb*+/-; *ARF*-/- (n=32), and *Rb*+/-; *ARF*+/- (n=13) mice show that *Rb*+/-; *ARF*-/- mice had a significantly decreased median survival of 168.5 days vs. 277 days for *Rb*+/- controls ($p<0.0001$). The *Rb*+/-; *ARF*+/- mice (n=13) exhibited a median survival of 262 days, comparable to that of *Rb*+/- mice ($p=0.34$) and significantly longer than that of *Rb*+/-; *ARF*-/- mice ($p<0.0001$).

mice appeared similar to 5-7 month old *Rb*+/- controls and were obvious upon histological examination. We examined comparably-sized tumors from these two populations in order to establish whether they were different with respect to their cellular content or growth characteristics. While grossly similar (compare Fig. 2A to 2B), BrdU labeling of cells in S phase, was significantly greater in the samples of *Rb*+/-; *ARF*-/- mice (Fig. 2D) relative to *Rb*+/- controls (Fig. 2C), indicating that these tumors had higher proportions of proliferating cells. Quantitation of the proliferation index (see Methods) for three matched pairs of tumor samples demonstrated that 15.3 ± 4% of nuclei in *Rb*+/-; *ARF*-/- tumors were labeled as compared to 2.3 ± 0.6 % ($p<0.05$) of nuclei for *Rb*+/- controls. This finding indicates that loss of *ARF* facilitates the proliferation of tumor cells that have lost *Rb*.

Alternatively, *ARF* mutation could accelerate tumorigenesis by abrogating an apoptotic response in cells that might otherwise have been eliminated. However, TUNEL staining of tumor sections isolated in this group of samples did not reveal any differences (data not shown). We also examined hematoxylin-eosin stained sections of these tumors to identify cells exhibiting hallmarks of apoptosis, including marginated chromatin and fragmented nuclei. Low levels of apoptosis were observed in all samples examined with no distinctive differences observed (data not shown). This evidence indicates that at this stage in tumor development, examining comparatively sized lesions, simultaneous inactivation of Rb and ARF provides tumor cells with a proliferation advantage without obvious effects on apoptosis.

Rb+/- (6.5mo)
Rb/ARF (4.5mo)
C
D

Figure 2. Comparison of tumors of similar stage from *Rb+/-; ARF-/-* and *Rb+/-* mice show higher levels of proliferation in *Rb+/-; ARF-/-* samples.

Mid-sagittal sections of pituitary tumors (P) at comparable stages of development were stained with hematoxylin and eosin (A, B) or used for BrdU immunohistochemistry (C, D). Samples from a (A) *Rb+/-* (6.5 months) mouse and a (B) *Rb+/-; ARF-/-* (4.5 months) compound mutant mouse are shown. These animals were injected with BrdU (10 mg/kg body weight) one hour prior to sacrifice and analysis of BrdU incorporation demonstrated a significantly higher S-phase fraction ($p<0.05$ for three matched pairs) in the *Rb+/-; ARF-/-* sample (D) relative to the *Rb+/-* control (C). Calibration bar is 100 mm for Figs. 2A and 2B, and 50 mm for 2C and 2D.

Rb+/-; ARF-/- mice develop early, aggressive focal lesions

To investigate the basis for the marked acceleration of tumor development in *Rb+/-; ARF-/-* mice, we analyzed serial sections of pituitary glands for early atypical proliferates (EAP) (14), the first morphologically distinct lesions that can be identified in *Rb+/-* animals. The abnormal cells that comprise EAPs often cluster at the border between the intermediate and posterior (neural) lobes, and are characterized by high nuclear-cytoplasmic ratios, irregularly-shaped nuclei, and coarse chromatin (14). *Rb+/-* mice develop the first EAPs between post-natal day (PND) 35 and PND 60 (14).

We dissected pituitary glands from PND 30 and PND 60 *Rb+/-*, *Rb+/-; ARF-/-*, and *Rb+/-; p53-/-* mice, and used hematoxylin-eosin-stained serial sections for our analysis. In our sample, none of the PND 30 *Rb+/-* or *Rb+/-; p53-/-* samples had any lesions, consistent with the interpretation that loss of *p53* does not accelerate the onset of pituitary tumorigenesis. Surprisingly, a number of PND 30 *Rb+/-; ARF-/-* samples had EAPs that were larger than the EAPs found in PND 60 *Rb+/-* controls (compare Fig. 3A to Fig. 3B; Table 2). Importantly, PCR analysis of micro-dissected EAPs demonstrated LOH at *Rb* showing that loss of *Rb* is still required for formation of tumors in *Rb+/-; ARF-/-* mice (Fig. 3F). The cells from EAPs show presence of the mutant allele only with loss of the wild-type allele (Fig. 3F). While all of the *Rb+/-* PND 60 EAPs were of Grade 1 or 2, 40% of the *Rb+/-; ARF-/-* PND 30 lesions were of Grade 4 (Table 2). Furthermore, by PND 60, the *Rb+/-; ARF-/-* mice had about three times as many independent EAPs on average (Figs. 3D, E; Table 1) as the *Rb+/-* (Fig. 3B) or *Rb+/-; p53-/-* (Fig. 3C)

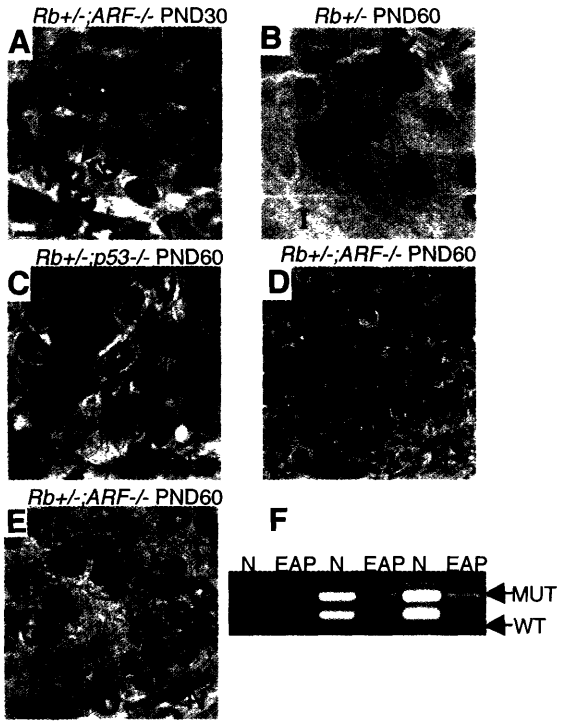

Rb+/-;ARF-/- PND30
A
Rb+/- PND60
B
Rb+/-;p53-/- PND60
C
Rb+/-;ARF-/- PND60
D
Rb+/-;ARF-/- PND60
E
F
N FAP N FAP N FAP
MUT
WT

Figure 3. Early Atypical Proliferates (EAPs) appear in the pituitaries of *Rb+/-; ARF-/-* compound mutants earlier than those in *Rb+/-* and *Rb+/-; p53-/-* compound mutant mice and are larger by PND 30.

Comparison of hematoxylin-stained EAPs from *Rb+/-*, *Rb+/-; ARF-/-*, and *Rb+/-; p53-/-* compound mutant mice. (A) EAP of a PND 30 *Rb+/-; ARF-/-* compound mutant mouse (1000X) showing dense groups of abnormal cells in the intermediate lobe (I) containing coarse chromatin, and large, irregular nuclei at the border with the posterior (neural) lobe. (B) EAP of a PND 60 *Rb+/-* mouse at the intermediate-neural lobe border (1000X). Arrows indicate abnormal cells. Note the size of this EAP relative to the lesion in (A). (C) EAP of a PND 60 *Rb+/-; p53-/-* compound mutant mouse showing a large lesion with densely packed abnormal cells in the intermediate lobe (1000X). (D) Low power view of an EAP of a PND 60 *Rb+/-; ARF-/-* compound mutant mouse (400X) showing typical large cluster of intermediate lobe (I) tumor cells near the neural lobe (N). (E) Higher power (1000X) view of one of the same lesions (D) demonstrating typical morphology of tumor cells. Note the presence of an apoptotic cell (arrow). (F) LOH at the *Rb* locus is observed in PND 60 EAPs from *Rb+/-; ARF-/-* mice. PCR analysis of DNA from laser capture dissected samples show retention of mutant allele with absence of wild-type allele in captured EAPs, (EAP) while both alleles are intact in adjacent normal pituitary (N). Calibration bar is 10 mm for Figs. 3A-C, E, and 25 mm for 3D.

Table 1: Early Atypical Proliferate Analysis in PND 30 and PND 60 mice

PND 30	Number of samples	Number of mice with lesions	Average number of lesions per affected mouse
Rb+/-	8	0	N/A
Rb+/-; ARF-/-	12	3	1.7 ± 0.4
Rb+/-; p53-/-	9	0	N/A

PND 60	Number of samples	Number of mice with lesions	Average number of lesions per affected mouse
Rb+/-	5	3	1.7 ± 0.7
Rb+/-; ARF-/-	5	5	7.0 ± 1.0
Rb+/-; p53-/-	5	4	2.8 ± 1.0

All EAPs from all affected mice were measured and classified according to tumor volume: Grade 1: $<10^3$ mm^3, Grade 2: 10^3–$5x10^4$ mm^3 , Grade 3: $5x10^4$–$1x10^5$ mm^3 , and Grade 4: $>10^5$ mm^3. Listed are the percentages and numbers in parentheses, for each tumor grade for mice of each genotype.

controls, and some were so large they had begun to fuse (these were counted as single lesions). While PND 60 *Rb+/-; p53-/-* samples and *Rb+/-* controls had comparable numbers of lesions (Table 1), the *Rb+/-; p53-/-* pituitaries contained nodules that were larger than the lesions found in *Rb+/-* controls (Fig. 3C; Table 2). Interestingly, while the PND 60 *Rb+/-; ARF-/-* mice had a significantly greater number of lesions than the PND 60 *Rb+/-; p53-/-* mice, the grade distributions of the EAPs overlapped for these two groups, indicating that loss of *p53* contributes to pituitary tumor development in *Rb+/-* mice, but that this effect appears to be mechanistically distinct from the effects observed with loss of *ARF*.

The great difference between the sizes of the PND 60 lesions makes it impractical to compare their apoptotic or proliferative indices. Careful examination of the large nodules in *Rb+/-; ARF-/-* samples revealed the presence of obvious apoptotic nuclei. Virtually all of the sections through each nodule contain apoptotic figures indicating that apoptosis is not completely compromised in these developing tumors in the absence of *ARF*. We cannot however rule out the possibility that the absence of *ARF* may protect cells from apoptosis prior to the development of early lesions that can be identified histologically.

The ARF locus is altered in tumors of Rb+/-; ARF+/- mice

Acquired proliferative advantage in developing tumors might provide a selection pressure to inactivate *ARF* in tumors of *Rb+/-* mice, perhaps making *ARF* loss an obligatory event in pituitary tumorigenesis. We addressed this possibility by examining whether *ARF* expression is affected in tumors of *Rb+/-* mice. Because *ARF* can behave as a classic tumor suppressor gene, reduction to homozygosity for the mutant allele may be selected for in lesions of *ARF+/-* mice as

Table 2: Size Distribution of Early Atypical Proliferates in PND 30 and PND 60 mice

GENOTYPE and AGE	Grade 1 $< 10^3$ μm^3	Grade 2 10^3–$5x10^4$ μm^3	Grade 3 $5x10^4$–$1x10^5$ μm^3	Grade 4 $>10^5$ μm^3
Rb+/- PND 60	40% (2)	60% (3)	0	0
Rb+/-; ARF-/- PND 30	40% (2)	20% (1)	0	40% (2)
Rb+/-; ARF-/- PND 60	22.9% (8)	51.4% (18)	2.9% (1)	22.9% (8)
Rb+/-; p53-/- PND 60	0	54.5% (6)	9.1% (1)	36.4 % (4)

EAPs were identified in hematoxylin-eosin stained serial sections of pituitaries isolated from Rb+/-, Rb+/-; ARF-/-, and Rb+/-; p53-/- mice at PND 30 and PND 60. Only EAPs that had distinct borders were counted as individual lesions.

previously reported (32). Despite the fact that *Rb*+/-; *ARF*+/- mice have a survival curve that is similar to that for *Rb*+/- mice (Fig. 1; p=0.34), two of six tumors from double heterozygotes demonstrated alterations at the *ARF* locus (Fig. 4). Southern blot analysis showed that all pituitary ("PIT") tumor samples exhibited LOH for *Rb* with only the band associated with the mutant allele remaining (Fig. 4A). DNA isolated from normal brain tissue ("Con") confirmed heterozygosity for the *Rb* mutation (9). Similarly, the same control samples, when analyzed with a probe specific for *ARF*, confirmed heterozygosity for the *ARF* mutation (Fig. 4B) (33). While four pituitary samples (Fig. 4B; "PIT": 8566, 8239, and data not shown) maintained equivalent levels of hybridization to both alleles of *ARF*, the mutant allele was enriched in two pituitary samples (Fig. 4B; "PIT": 8904, 8249). Western and northern blot analyses indicated that *ARF* is expressed in pituitary tumors from *Rb*+/- mice (Supplemental Information). These results, showing alterations in the *ARF* locus in two of six samples, indicate that while *ARF* loss may not be a requisite event in pituitary tumorigenesis, there is selective advantage for inactivating *ARF* in developing tumors.

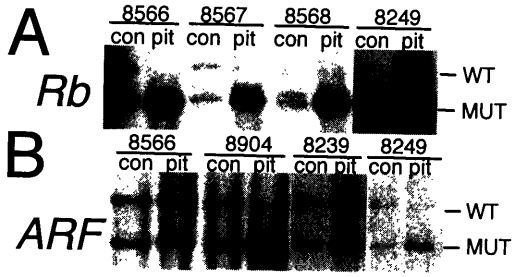
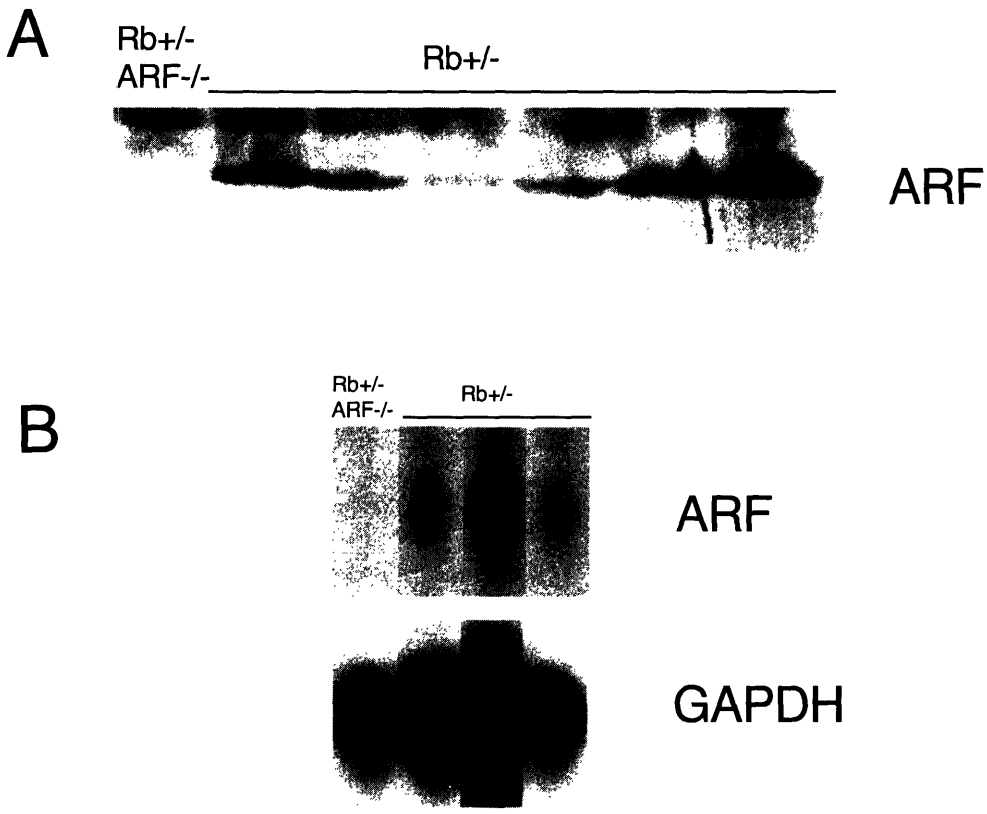

A
8566
8567
8568
8249
con pit con pit con pit con pit
Rb
WT
MUT
B
8566
8904
8239
8249
con pit con pit con pit con pit
ARF
WT
MUT

Figure 4. Tumors isolated from *Rb*+/-; *ARF*+/- animals exhibit alterations at the *ARF* locus. (A) DNA from normal adjacent cerebellum ("Con") confirms that the animals were heterozygous for *Rb*, and all samples of pituitary ("Pit") tumors showed LOH for *Rb* with only the 5.2 kB lower band associated with the mutant allele remaining. (B) DNA from normal adjacent cerebellum ("Con") confirmed that all the animals were heterozygous for *ARF*. Of four pituitary samples that also showed this pattern of equivalent hybridization, two are shown here ("PIT": 8566, 8239). Two pituitary samples exhibited alterations at the *ARF* locus. These samples demonstrated enrichment for the lower 6.0 kB band associated with mutant allele ("PIT": 8904, 8249).


A
Rb+/-
ARF-/-
Rb+/-
ARF
B
Rb+/-
ARF-/-
Rb+/-
ARF
GAPDH

Supplementary Figure 1. ARF is expressed in pituitary tumors of *Rb+/-* mice.

(A) Western blot of total protein (450μg) from pituitary tumor samples from moribund *Rb+/-* mice shows intact ARF expression (lanes 2 to 7). Included is a sample from *Rb+/-; ARF-/-* used as a negative control (lane 1). (B) Northern blot of pituitary tumor RNA from moribund Rb+/- mice shows ARF expression (lanes 2 to 4) which is absent in tumor from Rb+/-; ARF-/- animal (lane1). Blot was stripped and reprobed for GAPDH as a loading control.

DISCUSSION

The *Rb* pathway is critically important in pituitary tumorigenesis in mice. In addition to inactivation of *Rb*, mutations in upstream CKIs such as *p21Cip1*, *p27Kip1*, and *p18Ink4c* can also contribute to this process (17, 18, 22-25). Unlike mutants lacking genes that cooperate with loss of *Rb* in this process, *ARF*–deficient mice do not sustain pituitary lesions (32, 33), and evidence obtained in other systems suggested that *ARF* might play an important role downstream of *Rb* inactivation in tumorigenesis (44, 47-49). We probed this possibility directly by generating mice containing mutations in both *Rb* and *ARF*.

Our results demonstrate a marked acceleration of pituitary tumorigenesis in *Rb*+/- mice lacking *ARF*. Based on the analysis of early lesions, it is clear that the tumor suppressor function of *ARF*, while important, does not appear to act solely in a typical *p53*-dependent fashion. While it has been proposed that ARF connects the *Rb* and *p53* pathways through E2F activity and MDM2, respectively (3, 54, 55), other functions of ARF appear to be critical in pituitary tumorigenesis.

Rb+/-; *ARF*-/- compound mutants have significantly more early lesions at PND 30 and PND 60 than do *Rb*+/- or *Rb*+/-; *p53*-/- mice (Table 1). This early lesion analysis extends previous work in *Rb*+/-; *p53*-/- mice (15, 16). Nevertheless, *p53* loss has a measurable effect manifested in the significantly larger size of the lesions noted at that time point (Table 2). In addition, *Rb*+/-; *ARF*-/- compound mutants do not exhibit the pancreatic islet cell hyperplasia, pinealoblastoma, or bronchial epithelial hyperplasia previously reported in *Rb*+/-; *p53*-/- mice (15, 16), and at lower frequency, in *Rb*+/- mice (13, 15). In these tissues, *ARF* mutation does not recapitulate *p53*

inactivation, indicating that p53 may be regulated through ARF-independent mechanisms in these contexts.

The early lesion analysis has also demonstrated that EAPs can be detected as early as PND 30 in the *Rb+/-; ARF-/-* compound mutants but are absent in PND 30 *Rb+/-* and *Rb+/-; p53-/-* controls (14). By PND 60, *Rb+/-; ARF-/-* compound mutants have approximately three times as many EAPS that are also larger than those identified in the control populations (Table 1). Furthermore, PCR analysis of microdissected PND 60 EAPs from *Rb+/-; ARF-/-* animals demonstrate that loss of *Rb* is still required for tumor formation (Fig. 3F), even in the absence of *ARF*.

We propose this finding can be explained by at least three possible mechanisms. First, *ARF* could directly regulate pituitary development, for example by enforcing cell cycle arrest in differentiated melanotrophs; however, we have not observed any gross differences in the structures of pituitaries from mice lacking *ARF*, nor have we identified any lesions in adult *ARF*-deficient mice that would suggest such a role. Second, *ARF* deficiency may increase the proportion of individual cells that eliminate the remaining wild-type allele of *Rb* in development, or accelerate the timing of the loss of the wild-type allele of *Rb*. Conditional inactivation of *Rb* specifically in the pituitary resulted in mice with significantly shorter tumor latency than that of *Rb+/-* mice (21), as was observed with chimeric mice partly composed of *Rb* homozygous mutant ES cells (19, 20). Because the conditional *Rb* alleles were inactivated by an rPOMC-driven Flp enzyme (21), and POMC is expressed in midgestation (56), these results indicate that inactivation of both alleles of *Rb* earlier in development is sufficient to accelerate tumorigenesis. However, there is no evidence that *ARF* regulates the frequency of LOH events in tumorigenesis,

for example through increased frequency of sister-chromatid exchange or through a more general role in genomic instability independent of *p53*. Finally, *ARF* deficiency may enhance the survival or outgrowth of incipient tumor cells following the LOH event at the *Rb* locus. This proposal explains the findings here most efficiently in that it accounts for the fact that there are more individual lesions in *Rb+/-; ARF-/-* compound mutants as early as PND 30. If *ARF* deficiency can enhance the ability of these first *Rb*-deficient cells to survive or proliferate, then the number of histologically-apparent lesions that emerge would be increased.

Importantly, this hypothesis can also explain why there is incomplete selection against *ARF* in the *Rb+/-; ARF+/-* mice as LOH was detected by Southern blot in only 2/6 samples. Perhaps the temporal window within which *ARF* function is critical is quite limited and therefore incompatible with temporal requirements for LOH. The advantage conferred by the absence of *ARF* may be restricted to such a short period of time early in the development of tumors that selection is not maintained long enough to result in LOH. Alternatively, *ARF* loss would not be selected for in tumor cells if *ARF* acted in non-cell autonomous fashion to suppress tumorigenesis. The incomplete ARF LOH in Rb+/-;ARF+/- animals, and our findings that ARF can be detected at the RNA and protein level in tumors from Rb+/- animals suggests that complete loss of ARF is not required for pituitary tumor formation in Rb+/- animals. However we were technically limited in our ability to obtain an appropriate amount of normal pituitary intermediate lobe at an appropriate time in pituitary development to compare ARF levels to those in Rb+/- tumors. Therefore it remains possible that ARF levels may be reduced in tumors from Rb+/- animals and that ARF reduction may be important for pituitary tumorigenesis in Rb+/- mice.

Our evidence in more advanced tumors of 4-7 month-old mice indicates that the tumors from *Rb+/-; ARF-/-* mice have higher proliferation indices than do tumors at similar stages in *Rb+/-* controls (Fig. 2). Thus *ARF* loss likely contributes to tumor development by also enhancing the ability of cells within well-developed tumors to proliferate.

We have shown that mutation of *ARF* significantly accelerates pituitary tumor development in *Rb+/-* mice. Because *ARF* loss has such a dramatic effect, particularly on the number of early lesions that is not observed with *p53* loss, *ARF* may be regulating a *p53*-independent mode of tumor suppression. Given the role of ARF in inhibiting MDM2 (55), it is possible that the *p53*-independent functions of MDM2 are being regulated by ARF. For example, MDM2 can inhibit the transactivation function of the p53-related protein p73, which has been shown to be important in a variety of *p53*-independent apoptotic pathways (57-59). In addition, MDM2 has been shown to potentiate E2F-1-mediated transactivation and cellular proliferation (60, 61). Even in the absence of *Mdm2*, it has been shown that loss of both *ARF* and *p53* can cooperate in the development of novel tumors (62), and prolonged exposure to MYC in B cell lymphomas provides selection for overexpression of *Mdm2* in the absence of *ARF* (63). Therefore, it is likely that even in the absence of a requirement to inactivate *p53* in pituitary tumorigenesis, ARF may be restraining some other activity of MDM2 or acting independently of the *Mdm2-p53* pathway to inhibit tumor development. The finding that ARF expression can enforce a cell cycle arrest in *Mdm2/p53* double mutant mouse embryo fibroblasts (MEFs) but not in *p53-/-* MEFs is evidence for novel targets of ARF (62). There is also evidence that ARF can induce apoptosis in

the absence of *p53* (64). In vivo, *ARF*-/- mice have defects in eye development that are not observed in p53-/- mice (50).

Our analysis is the first in vivo demonstration that *ARF* plays an important role in tumor suppression in the context of *Rb* inactivation. The early lesion analysis suggests that *ARF* loss does not completely compromise apoptosis since dying cells can be readily detected in PND 60 *Rb*+/-; *ARF*-/- nodules. However, it is not possible to know whether *ARF* promotes apoptosis immediately following loss of the wild-type allele of *Rb* as this event occurs prior to the appearance of histologically recognizable lesions. Later in tumor development, it is clear that loss of *ARF* can increase the proportion of tumor cells that are proliferating. These data help to define a broad scope of effects of the *ARF* tumor suppressor in the context of *Rb* inactivation that extends beyond functional inactivation of *p53*.

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