ANALYSIS OF THE ROLE OF THE RETINOBLASTOMA SUSCEPTIBILITY GENE IN MURINE DEVELOPMENT AND TUMORIGENESIS

by

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ABSTRACT

Studies on the retinoblastoma susceptibility gene (*RB*) have played a central role in the conceptual development of the tumor suppressor gene field. Patients inheriting a mutation in the *RB* gene develop the rare pediatric eye cancer retinoblastoma with 90% penetrance. In addition, somatic mutations in the *RB* gene have been found in many sporadically occurring human tumors. A great deal of insight has also been gained from studies of retinoblastoma protein (pRB) in tissue culture. These studies have shown that the protein product plays an important role in cell cycle coordination, perhaps by modulating some aspect of the restriction point during the G1 phase of the cell cycle. Despite this vast amount of information, until recently very little was known about the normal function of *RB* in development. Techniques developed in the last decade have allowed mice with targeted mutations to be created. Several groups have exploited these techniques to create mice with germline inactivating mutations in the murine version of the retinoblastoma susceptibility gene (*Rb*). Initial analysis of these mice indicated that heterozygotes did not develop retinoblastoma, instead they develop tumors in the intermediate lobe of the pituitary and in the thyroid. Mice homozygous for the mutant allele die between 13.5 and 15.5 days of gestation with associated defects in hematopoiesis and neurogenesis.

The focus of this work has been on expanding the use of these mice to better understand *RB* function. One surprising observation is the relatively limited tumor spectrum observed in both mice and humans which are constitutionally heterozygous for an *RB* mutation. A potential explanation for this is that other genetic alterations must occur in combination with *Rb* mutation to cause tumorigenesis in most tissues. To test this model, *Rb*-deficient mice were crossed with a *p53* mutant strain. Animals carrying mutations in both genes are susceptible to a wider spectrum of tumors. A potential mechanism for this cooperativity is presented which shows that at least in one system (the developing murine lens) *Rb*-deficiency leads to *p53*-dependent apoptotic cell death. This apoptotic induction could serve to eliminate potentially transformed cell from the organism.

One limitation to the utility of the *Rb*-deficient strain was the attendant embryonic lethality. To bypass this lethality and assess the effects of *Rb*-deficiency during later stages of development and in adult mice, ES cells were created which carried targeted mutations in both copies of the *Rb* gene and used them to create chimeric animals. Analysis of tissue homogenenates from these chimeras showed that *Rb*-deficient cells could contribute to most tissues of the adult mouse without pathological consequences. To further this analysis, *Rb*-deficient ES cells have been created which carry a transgene directing nearly ubiquitous expression of B–galactosidase. Staining of chimeric tissues derived from these ES cells for B–galactosidase activity has enabled us to identify and analyze *Rb*-deficient cells on a single cell level within these chimeras.

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Figures and tables within:

Lee MH, Williams BO, Mulligan GJ, Mukai S, Bronson RT, Dyson N, Harlow E, and Jacks T. (1996) Targeted disruption of *p107*: evidence for functional overlap with *Rb*. Genes and Development (in press).

APPENDIX D

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ABBREVIATIONS USED

B–gal	Beta galactosidase
BrdU	Bromo-deoxyuridine
DNA	Deoxyribonucleic acid
ES cells	Embryonic stem cells
FACS	Fluorescent activated cell sorter
FdU	Fluoro-deoxyuridine
G418	A synthetic analog of neomycin
GPI	Glucose phosphate isomerase
$hygro^R$	Gene conferring resistance to hygromycin
kd	Kilodalton
Lac Z	Beta galactosidase gene
LOH	Loss of heterozygosity
MEFs	Murine embryonic fibroblasts
mg	milligram
ml	milliliter
neo^R	Gene conferring resistance to neomycin or G418
pRb	Retinoblastoma susceptibility protein
RB	Retinoblastoma susceptibility gene (human)
Rb	Retinoblastoma susceptibility gene (mouse)
Rb-DKO Chimeras	Chimera derived from injection of Rb-deficient ES cells into blastocysts (Rb Double Knock Out)
Rb-DKO ES cells	ES cells with both copies of the RB gene mutated
ROSA-26	Reverse orientation splice acceptor (Lac Z transgenic mice)
X-gal	5-Bromo-3-chloro-indolyl-galactoside

NOMENCLATURE

I have used the accepted nomenclature in referring to genes and proteins throughout this manuscript. Gene names are italicized while the same abbreviation referring to the protein product of the gene is not. In addition, when the gene referred to is a mouse gene (and where it is applicable) the gene abbreviation is written as the first letter being capitalized and all subsequent letters being lowercase (*Rb* and *Apc*). When the human counterpart is referred to all the letters in the abbreviation are capitalized (*RB* and *APC*).

CHAPTER 1

Tumor Suppressor Genes

The discovery and characterization of oncogenes in the late 1970s and early 1980s revolutionized the study of cancer (Bishop, 1995). Cancer could now be considered as a genetic disease in which mutations in molecularly definable genes could be linked to tumor development. Most oncogenes were originally isolated either because their coding sequences had been coopted by retroviruses to give them transforming potential or because their addition to normal cells caused normal growth control mechanisms to be compromised leading to transformation (Bishop, 1985; Bishop, 1995; Land et al., 1983; Ruley, 1983; Shih et al., 1981; Shih and Weinberg, 1982; Stehelin et al., 1976).

Because the carcinogenic effects of tumor suppressor gene mutations were usually the result of loss of function mutations in both cellular copies of the gene (Lee et al., 1987; Levine, 1993), they proved more challenging to isolate and characterize. The existence of tumor suppressor genes was originally postulated on the basis of somatic cell fusion experiments in which some fusions between transformed and normal cells lead to an inhibition of the transformed cell phenotype (Harris et al., 1969). This observation was later expanded to show that the introduction of small regions of individual chromosomes from normal cells could suppress the transformed properties of some tumor cells (reviewed in Stanbridge, 1990). These data, along with the seminal work of Knudson (described in Chapter 2) helped establish the concept that cancer could result from the loss of negative growth control in addition to gain of function mutations in growth-promoting oncogenes. The development of positional cloning methods in the 1980s has facilitated the identification of many tumor suppressor genes to date.

Almost all of the tumor suppressor genes identified to date have been cloned on the basis of inherited mutations in these genes segregating with an obvious familial cancer predisposition. The prototypic example of the identification of the retinoblastoma susceptibility gene (*RB*) will be described in detail. At least ten other tumor suppressor gene mutations identified by their association with familial cancer syndromes have been described. Like the products of oncogenes, the protein products of these genes participate in a wide spectrum of cellular functions. The

functions of these genes and the inherited cancer syndromes associated with them are summarized in Table 1-1. Besides the *RB* gene, this work has focused heavily on the activities of the *p53* gene. Because of this, the identification and activities of the *p53* gene will also be reviewed in detail.

Table 1-1. Functions of human tumor suppressor genes and tumors associated with their inactivation (Adapted from Williams and Jacks, 1996)

Gene	Protein Features	Inherited Syndromes (Human)#	Associated Cancers (Human)#
RB	Nuclear phosphoprotein, Binds transcription factors	Familial retinoblastoma	Retinoblastoma; osteo-sarcoma; breast, lung, and bladder carcinoma
p53	Nuclear phosphoprotein, Transcription factor	Li-Fraumeni syndrome	Several (50% of all cancer)
Wt-1	Zinc fingers, Transcription factor	Wilms tumor	Nephroblastoma
APC	Coiled-coil motif, Binds B-catenin	Familial adenomatous polyposis coli, Turcot's syndrome	Colon and Brain
NF-1	ras-GAP activity	Neurofibromatosis Type I	Neurofibromatosis, Colon carcinoma, Astrocytoma
NF-2	Membrane cytoskeletal attachment	Neurofibromatosis Type II	Vestibular Schwannoma, Meningioma, Ependymoma
$p16^{MTS1}$	Cyclin/cdk inhibitor	Familial melanoma	Several
DCC	N-CAM homology	??	Colon
VHL	Inhibits transcriptional elongation	von-Hippel-Lindau syndrome	Renal cell carcinoma, pheochromocytoma
BRCA1	Zinc finger, secreted granin ?, nuclear protein ?	Familial breast/ovarian cancer	Breast and ovarian
BRCA2	granin ?	Familial breast/ ovarian cancer	Breast and ovarian

References or reviews for the information summarized in Table 1 are as follows: *RB* (Hollingsworth et al., 1993; Hollingsworth, 1993; Riley et al., 1994; Weinberg, 1992; Weinberg, 1995) and below, *p53* (Greenblatt et al., 1994; Levine, 1992; Levine et al., 1991) and below, *WT-1* (Hastie, 1994), *APC* (Miyaki et al., 1995; Polakis, 1995), *NF-1* (McCormick, 1995; Vishochil et

al., 1993), *NF-2* (Gusella et al., 1996), *p16^{MTS1}* (Harper and Elledge, 1996), *DCC* (Cho and Fearon, 1995), *VHL* (Duan et al., 1995a; Duan et al., 1995b; Kibel et al., 1995), *BRCA1* (Holt et al., 1996; Jensen et al., 1996; Miki et al., 1994), and *BRCA2* (Jensen et al., 1996; Tavtigian et al., 1996; Wooster et al., 1995).

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CHAPTER 2

The Retinoblastoma Gene

Identification and cloning of the retinoblastoma susceptibility gene

Retinoblastoma is a rare tumor of the retina which develops in approximately 1 in 20,000 children in the United States each year (Dryja, 1989; Dunphy, 1964; Weinberg, 1992). It was first reported in the early nineteenth century, and in those relatively primitive medical times was almost always fatal (Dryja, 1989; Dunphy, 1964; Weinberg, 1992). However, better methods of treatment allowed survival of affected individuals, and around the turn of the century vertical transmission of the disease was reported (DeGouvea, 1886; Dryja, 1989; Dunphy, 1964; Ridley, 1905; Weinberg, 1992). As more such cases were examined it became evident that retinoblastomas occurring in familial clusters differed from those occurring sporadically. The inherited cases were much more likely to occur earlier in life and present as multifocal and bilateral. In 1971, Alfred Knudson published an analysis of the two different forms of the disease which came to several conclusions that would lay the conceptual framework for the discovery and analysis of tumor suppressor genes (Knudson, 1971). Knudson suggested the difference between the two types of retinoblastoma was that in the sporadic cases two relatively rare events needed to occur to initiate tumor development, while in the inherited cases only one event was necessary. Knudson later extended this "two-hit" hypothesis to explain the differences between the familially associated and sporadic cases of Wilms tumor, a childhood cancer of the kidney (Knudson and Strong, 1972).

The concept that inactivating mutations in both alleles of a single gene could explain the "two-hits" proposed by Knudson was first advanced by Comings (Comings, 1973). Experimental support for this idea began to emerge in 1978 when Yunis and Ramsey described the deletion of an area of chromosome 13 (band q14) in some DNA samples derived from retinoblastomas (Yunis and Ramsey, 1978). Five years later, several groups reported that retinoblastoma patients who were constitutionally heterozygous for DNA markers in the 13q14 region underwent reduction to homozygosity in this region in their tumor DNA (Bendedict et al., 1983; Cavenee et al., 1983; Dryja et al., 1984; Godbout et al., 1984; Sparkes et al., 1983). Shortly thereafter, the cloning of the

gene underlying retinoblastoma development was reported by Friend et al. (Friend et al., 1986) and later confirmed by other groups (Fung et al., 1987; Lee et al., 1987b). The gene was found to span approximately 200 kilobases of DNA in the 13q14 region (Hong et al., 1989; McGee et al., 1989). Further analysis revealed that the gene contained 27 exons ranging in size from 31 to 1889 nucleotides (Hong et al., 1989; McGee et al., 1989). In addition, detailed examination of the promoter region of the gene showed it to be GC rich and contain binding sites for the transcription factors ATF, Sp-1, and E2F (reviewed in Riley et al., 1994). While the initial isolation of the gene was dependent on the analysis of individual tumors from patients who had inherited large deletions in the gene, it was subsequently found that a large number of retinoblastomas were actually associated with point mutations in the *RB* gene (Canning and Dryja, 1989; Dunn et al., 1988; Fung et al., 1987; Gallie et al., 1990; Horowitz et al., 1989; Kaye et al., 1990; Lee et al., 1987b; Yandell et al., 1989). The end result of all the point mutations was to create functionally inactive pRB. The significance of the promoter binding sequences is highlighted by the fact that several examples of familially-associated retinoblastoma have been associated with deletions or point mutations within these binding sites (Bookstein et al., 1990a; Sakai et al., 1992).

After the gene was cloned, several groups screened DNA from a variety of different tumor types to examine whether *RB* function was lost via deletion or point mutation. Surprisingly, even though patients who inherit a defective *RB* allele are not significantly predisposed to any tumors besides retinoblastoma (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987b), osteosarcoma (Friend et al., 1986), and perhaps pinealoblastoma (Jakobiec et al., 1977; Pesin and Shields, 1989; Stannard et al., 1985), somatic mutations in the *RB* gene were shown to occur in several other tumor types. These included some types of sarcomas (Friend et al., 1987; Shew et al., 1989; Stratton et al., 1990) as well as carcinomas of the cervix (Scheffner et al., 1991), prostate (Bookstein et al., 1990b), bladder (Cairns et al., 1991; Ishikawa et al., 1991; Takahashi et al., 1991), breast (Lee et al., 1988; T'Ang et al., 1988; Varley et al., 1989), and lung (Hensel et al., 1990; Ookawa et al., 1993; Rygaard et al., 1990; Yokota et al., 1988). In addition, mutations affecting other genes involved in the regulation of RB protein (pRB) activity have been estimated

to occur in a still larger spectrum of human tumors (discussed below). The data implied that pRB played a potentially important role in growth control in many cell types besides retinoblasts.

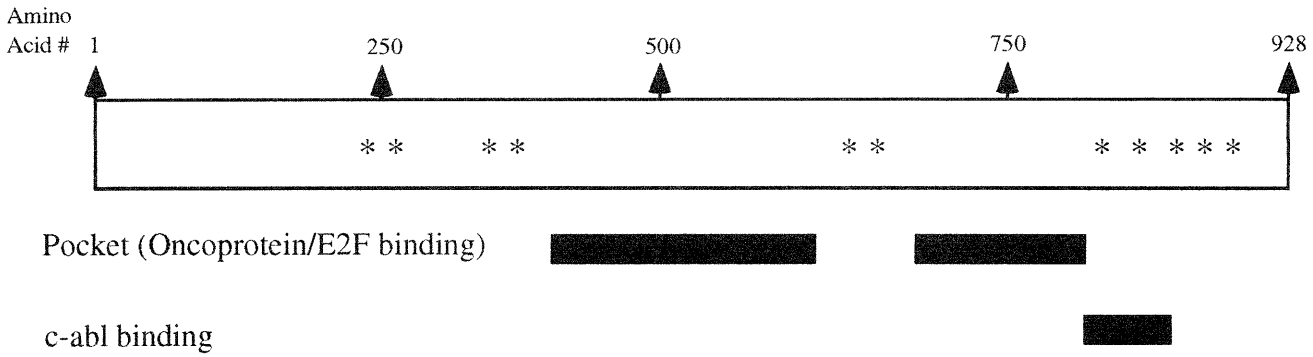
In vitro evidence that the *RB* gene can directly suppress cell proliferation

The first direct evidence that *RB* could suppress transformation and tumorigenesis came from studies in which *RB* was reintroduced into cell lines which were deficient for *RB* as a result of mutation (reviewed in Riley et al., 1994). In several cases such reintroduction was shown to have effects including the inhibition of cell proliferation *in vitro*, inducing changes in cellular morphology, reducing the growth potential of cells in soft agar, and inhibiting the growth of tumor cell lines upon injection into nude mice. Examples of tumor cell lines which are affected by the reintroduction of *RB* include those derived from retinoblastomas (Huang et al., 1988; Medraperla et al., 1991; Xu et al., 1991), osteosarcomas (Huang et al., 1988), and carcinomas of the breast (Wang et al., 1993b), bladder (Goodrich et al., 1992; Takahashi et al., 1991), and prostate (Bookstein et al., 1990b). Assays based on these systems, especially the ability of *RB* to induce cell cycle arrest and morphological changes in the osteosarcoma cell line SAOS-2 (Hinds et al., 1992; Qian et al., 1992), have been heavily utilized to assess aspects of *RB* function (see below).

Analysis of the *RB* gene product

The cDNA derived from the *RB* gene is 4757 nucleotides long and contains an open reading frame predicted to encode a protein containing 928 amino acids (reviewed in (Riley et al., 1994; Weinberg, 1992). Northern and subsequent Western blot analysis revealed that the gene was expressed in an almost ubiquitous fashion (Bernards et al., 1989; Lee et al., 1987a). The predicted molecular mass of the protein was 105 kD. However, initial analysis of pRB sequence provided few clues to its potential function. A schematic representation of the domains discussed below is shown in Figure 2-1.

Figure 2-1. Characteristics of pRB



* Denotes a serine or threonine within located in a cdk consensus recognition site. Residues are 249, 252, 356, 373, 608, 612, 788, 795, 807, 811, and 821. Residue 249 is not a perfect consensus site but has been shown to be phosphorylated in vitro by cdc2 (Lees et al., 1991).

The protein was first identified by Wen-Hwa Lee and colleagues in 1987 with antibodies raised against the RB protein derived from expression in *E. coli* (Lee et al., 1987a). Initial analysis revealed that the protein was present at all phases of the cell cycle suggesting that its activity was regulated post-translationally (Chen et al., 1989; Lee et al., 1987a). Western blot analysis of cell extracts with this antibody revealed that it recognized a number of proteins migrating between 110 and 116 kD (Ludlow et al., 1990; Shew et al., 1989). Subsequent analysis showed that these phosphopeptides were phosphorylated exclusively on serine and threonine residues (Chen et al., 1989; DeCaprio et al., 1988; DeCaprio et al., 1989; Furukawa et al., 1990; Lees et al., 1991; Lin et al., 1991; Ludlow et al., 1990; Shew et al., 1989). It was later shown that many of these residues were phosphorylated by the activity of cdk family members based on the observations that purified cdc2 could phosphorylate pRB *in vitro* on many of the same sites that they were phosphorylated *in vivo* (Lees et al., 1991; Lin et al., 1991; Taya et al., 1989). Furthermore, the first five of the *in vitro* phosphorylated sites mapped were found to contain a consensus cdc2 recognition motif (Lees et al., 1991). Much *in vivo* and *in vitro* evidence now supports the model that cyclin dependent kinases are the major, if not only, kinases involved in pRB phosphorylation (described below).

The pattern of pRB phosphorylation oscillates during different stages of the cell cycle (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1988; DeCaprio et al., 1989; Furukawa et al., 1990; Mihara et al., 1989). During G0 and early to mid G1, hypophosphorylated forms of the protein predominate. As the cell progresses through the restriction point late in G1, the protein becomes highly phosphorylated probably due to cdk/cyclin kinase activity (discussed below). These highly (or hyper-) phosphorylated forms of pRB remain through S, G2, and most of M phase. The protein then begins to become dephosphorylated again during anaphase. This process then is completed during early G1. Relatively little is known about the mechanisms underlying the dephosphorylation of pRB. However, the facts that pRB was shown to interact with the catalytic subunit of phosphoprotein phosphatase type 1 (PP-1) (Durfee et al., 1993) and that inhibitors specific for PP-1 block pRB dephosphorylation (Hollingsworth, 1993) suggest that PP-1 may control it.

The *RB* gene product interacts with several DNA tumor virus oncoproteins

All DNA tumor viruses need to replicate their own viral genome to propagate and survive. Large DNA tumor viruses (such as herpes simplex virus) typically encode most of the enzymes necessary for this process and need only minor components of the cellular environment for its completion (Knipe, 1989) On the other hand, some of the smallest DNA tumor viruses, such as parvoviruses, have evolved the opposite replication strategy. These viruses are heavily dependent on cellular factors for all aspects of viral replication and thus only replicate their DNA when the cellular DNA replicates (reviewed in Dyson and Harlow, 1992).

Several DNA tumor viruses (most notably adenoviruses, papillomaviruses, and polyomaviruses) have evolved strategies which lie between these two extremes (Dyson and Harlow, 1992). These viruses are still heavily dependent on cellular factors, but instead of merely waiting for DNA replication to occur, their genomes encode proteins which help stimulate infected cells to enter S phase. These proteins include the E1A protein produced by adenovirus, the E7 protein encoded for by the papillomavirus genome, and the large T antigens of polyomaviruses.

The E1A protein are the first polypeptides expressed from the viral genome following adenovirus infection and have been shown to have a number of different functions (Lewis and Mathews, 1980; Nevins, 1981). In order to gain further insight into the mechanisms underlying E1A function, two groups used antibodies derived against the E1A protein to identify cellular proteins which interacted with E1A (Harlow et al., 1986; Yee and Branton, 1985). Remarkably, one protein which coimmunoprecipitated with E1A was pRB (Whyte et al., 1988). Further analysis showed that was a direct interaction. Stimulated by this observation, DeCaprio et al. showed that a polyomavirus protein, the simian virus 40 (SV40) large T antigen, could also form a complex with the RB protein (DeCaprio et al., 1988). Subsequently it was shown that the E7 oncoproteins of

human papillomaviruses and large T antigens of other polyomaviruses could also interact with pRB (DeCaprio et al., 1988; Dyson et al., 1990; Dyson et al., 1989; Munger et al., 1989).

Since E1A (Dyson and Harlow, 1992; Houweling et al., 1980; Land et al., 1983; Ruley, 1983) (as well as SV40 large T (Livingston, 1992) and E7 (Munger et al., 1992)) could act as oncogenes and loss of *RB* function was associated with the development of human tumors, a simple hypothesis was forwarded that the binding of these proteins to pRB resulted in pRB functional inactivation (Dyson and Harlow, 1992; Livingston, 1992; Munger et al., 1992). This would create a situation equivalent to that seen in cells which had lost *RB* function via mutation. Importantly, the hypophosphorylated forms of pRB were the only ones targeted by the viral oncoproteins suggesting that they were the functionally active forms (Dyson and Harlow, 1992; Livingston, 1992; Munger et al., 1992). Such functional inactivation of the growth suppressing RB protein would help drive the infected cell into S phase and facilitate viral replication.

This model of viral oncoprotein and pRB function has been supported by several lines of experimental work which themselves have revealed important insights into *RB* function. First, the regions of the viral oncoproteins which mediate binding to pRB are necessary for full viral activity (Dyson and Harlow, 1992; Livingston, 1992; Munger et al., 1992). In addition, the regions of pRB which bind to the viral oncoproteins (referred to as the "pocket" region) have been shown to be favored sites for point mutations associated with human tumors (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990). This implied that the viral oncoproteins were targeting a region of pRB which normally acted in a growth inhibitory manner.

The *RB* gene product binds to E2F, a cellular transcription factor

Work during the mid-1980s had shown that the E1A could dissociate complexes containing the cellular transcription factor E2F resulting in the release of free E2F (Bagchi et al., 1990; Nevins, 1992). E2F was originally identified as a component of the adenovirus E2 promoter transcription

complex (Nevins, 1992) and has subsequently been shown to have the ability to stimulate cellular proliferation and transactivate promoters of many genes which are thought to play a role in stimulating cell cycle progression. A partial list of genes which contain putative E2F target sites in their promoters include *dihydrofolate reductase* (Blake and Azizkhan, 1989; Hiebert et al., 1991), *thymidine kinase* (Kim and Lee, 1991), *DNA polymerase* α (Pearson et al., 1991), *c-myc* (Hiebert et al., 1989; Thalmeier et al., 1989), *N-myc* (DePinho et al., 1986; Mudryj et al., 1990), *cdc2* (Dalton, 1992), *B-myb*(Lam and Watson, 1993), *PCNA* (Lee et al., 1995a), and *cyclin E* (Ohtani et al., 1995).

Since E1A both bound pRB and caused the release of free E2F from higher order complexes, several groups tested whether binding of E1A released pRB from an interaction with E2F which allowed E2F to perform its cellular functions. In 1991, four groups reported that this indeed was the case (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991). E2F was shown to bind to hypophosphorylated form of pRB and be released upon E1A binding to pRB. The binding site for E2F within the pRB protein was found to map partially to the E1A-binding site (the pocket) and also to another area C-terminal to the pocket (see Figure 2-1).

The simple model that emerged from these studies was that pRB normally inhibits cell cycle progression by binding to and negatively regulating the activity of E2F complexes (Nevins, 1992). While in general this model has proven to be accurate, it has become necessarily more complex due to several observations in recent years. A great deal of accumulating evidence suggests that besides simply negatively regulating E2F activity, pRB may actually repress transcriptional activity from certain promoters via its association with promoter sites through its E2F interaction (since E2F complexes have specific DNA binding activity) (Weintraub et al., 1995; Weintraub et al., 1992). To add another level of complexity to the equation, E2F has been shown to be a dimeric complex between one E2F and one DP subunit (reviewed in (La Thangue, 1994). The first E2F subunit, E2F-1 was cloned in 1992 . Subsequently, the identification of four other E2Fs (E2F 2-

5) has been reported (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Ivey-Hoyle et al., 1993; Lees et al., 1993; Sardet et al., 1995). In addition, multiple genes encoding for DP subunits have also been identified, although relatively little has been reported about their functions (Girling et al., 1994; Girling et al., 1993).

Consistent with the model that pRB inhibits cell cycle progression, overexpression of E2F-1 has been shown to be sufficient to drive arrested cells into S phase (Johnson et al., 1993). Furthermore, SAOS-2 cells overexpressing E2F-1 are able to overcome the block in cell cycle progression induced by pRB introduction (Qin et al., 1995).

As discussed below, the different E2F subunits appear to confer specificity of each complex to associate differentially with different pRB-related proteins. Whether the different E2F subunits (or even the DP subunits) direct E2F complexes to specific promoters remains to be determined.

pRB can interact with a number of cellular proteins

Besides its binding to E2F complexes, pRB has been reported to interact, at least *in vitro*, with several other cellular proteins. In general, these proteins appear to interact preferentially with the hypophosphorylated form of pRB. A partial list of these proteins includes the transcription factors c-myc (Rustgi et al., 1991), c-abl (Welch and Wang, 1993), elf-1 (Wang et al., 1993a), Id-2 (Iavarone et al., 1994), myoD (Gu et al., 1993), myogenin (Gu et al., 1993), PU.1 (Hagemeier et al., 1993), ATF-2 (Kim et al., 1992), UBF (Cavanaugh et al., 1995), and BRG-1 (Dunaief et al., 1994) as well as D-type cyclins (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993), the protein phosphatase PP1-α2 (Durfee et al., 1993), mdm2 (Xiao et al., 1995), and the human homolog of a yeast ras regulatory protein MSI1 (Qian et al., 1993). The importance of these individual interactions or their physiological relevance in modulating pRB function is not clear in many cases.

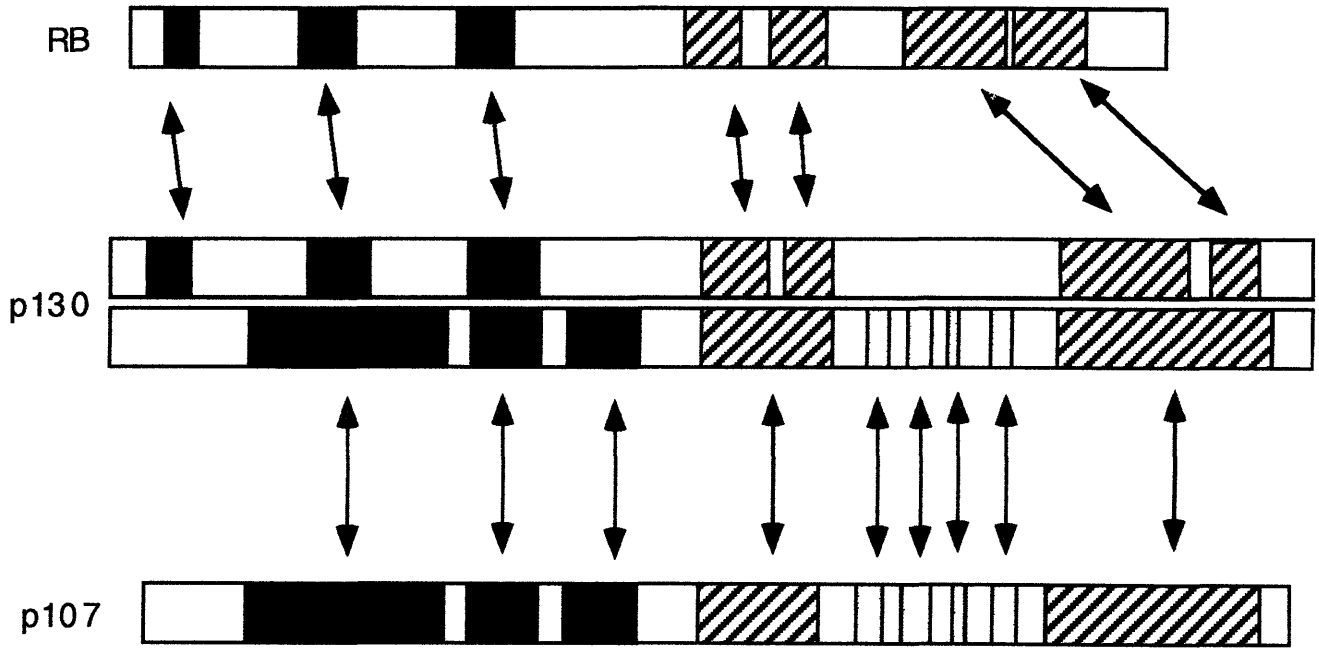
Extending the concept of the pRB:E2F interaction, a model that has been suggested based on the ability of pRB to interact with this plethora of cellular transcription factors is that pRB acts as a gatekeeper for the initiation of cell cycle progression (Hollingsworth et al., 1993; Hollingsworth, 1993; Riley et al., 1994; Weinberg, 1995). In the hypophosphorylated state it binds to and inhibits a number of these transcription factors. Upon stimulation of cell cycle progression by external signals, the machinery to phosphorylate pRB is activated resulting in hyperphosphorylated pRB and the coordinated release and activation of these transcription factors. The fact that pRB becomes hyperphosphorylated coincident with the restriction point (Weinberg, 1995), the point after which mammalian cells are committed to going through the cell cycle to the next G1 phase (Pardee, 1989), further supports this model.

RB is a member of a multigene family

Work on DNA tumor virus oncoproteins also impacted the retinoblastoma field in another significant way in helping establish that *RB* is actually one of a three member gene family which share significant functional and structural homology. The work which lead to this understanding was based again on studies of proteins which could be shown to interact with DNA tumor virus oncoproteins by coimmunoprecipitation. Some of the same mutant versions of E1A (as well as large T antigen) which had lost the ability to interact with a number of the other coimmunoprecipitating proteins (Dyson and Harlow, 1992). Among these were proteins referred to by their estimated molecular weight, p107 and p130. The genes encoding for p107 (Ewen et al., 1991) and p130 (Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993) have now been cloned. The three family members share considerable homology (see Figure 2-2 and reviewed in (Cobrinik, 1996).

Figure 2-2.

Structural Homology Between Members Of The *Rb*-gene Family



Homology whose functional relevance is unknown

Homology in the E1A/large T/E7/E2F binding site

Homology between the spacer regions (cdk/cyclin binding site) of p107 and p130

(Adapted from Li et al., 1993)

Further analysis of the protein products of these genes has confirmed that they do share functional characteristics with pRB. Besides the shared ability to bind to DNA tumor virus oncoproteins which was the basis for their identification, the proteins also bind to members of the E2F family of transcription factors with pRB having been shown to bind to E2F complexes containing E2F-1, -2, -3, or -4 (Bagchi et al., 1991; Chellappan et al., 1991; Fagan et al., 1994; Helin et al., 1993; Helin et al., 1992; Hiebert et al., 1992; Horio et al., 1993 ; Kaelin et al., 1992; Krek et al., 1993; Lees et al., 1993; Moberg et al., 1996) and p107 and p130 binding to complexes containing E2F-4 or -5 *in vivo* (Beijersbergen et al., 1994; Hijmans et al., 1995; Moberg et al., 1996; Sardet et al., 1995; Vairo et al., 1995). The distribution of these complexes has been shown to change during progression through the cell cycle (Chittenden et al., 1993; Cobrinik et al., 1993; Moberg et al., 1996; Schwarz et al., 1993; Shirodkar et al., 1992). p130:E2F complexes make up the majority of E2F complexes during G0 and early G1. During mid to late G1, pRB becomes the major E2F binding partner, while during late G1 and S p107 becomes the predominant binding partner.

Further support for grouping these gene products into a gene family comes from analysis of the cellular effects of reintroduction or ectopic expression of these genes. As briefly mentioned above, the introduction of the retinoblastoma gene into several cell types suppressed aspects of the transformed phenotype. Introduction of *p107* and *p130* into tumor cell lines has shown that they also have the ability to suppress aspects of the transformed phenotype when overexpressed. However, the mechanism underlying this suppression appears to differ among the *RB*-family members. For example, while the ability of *RB* to suppress colony formation when introduced into SAOS-2 cells can be overcome by overexpression of E2F-1, while the ability of *p107* to suppress colony formation is unaffected (Zhu et al., 1993). In addition, some cell lines are differentially sensitive to suppression by pRB-family members. For example, colony formation and proliferation in the cervical carcinoma cell line C33-A is not inhibited by the introduction of *RB*, but it inhibited by *p107* (Zhu et al., 1993). Another example is the glioblastoma cell line T98G in which colony formation is inhibited by *p130* but not by *p107* or *RB* (Claudio et al., 1994).

The suggestion that *p107* and *p130* play roles in suppressing aspects of cellular transformation is difficult to reconcile with other observations. The most important of which is that despite great efforts to identify human tumors carrying mutations in *p107* and/or *p130*, no evidence for any such mutations has been reported. It should be noted, however, that it has been suggested that *p130* may be a mutational target during the development of nasopharyngeal tumors in Chinese garment workers (Claudio et al., 1994; Mascola et al., 1992). Whether *p130* will be the important target of these mutational events remains to be seen. In addition, the creation of mice carrying targeted mutations in the *p107* and *p130* genes (see Appendices C and E) has revealed that mice deficient for either one of these genes fail to show any tumor predisposition (see Appendix C and (Cobrinik et al., 1996)). Extending this even further, mice heterozygous for a mutation in one of the genes and completely deficient for the other (either *p107*+/-;*p130*-/- or *p107*-/-;*p130*+/-) still do not display any predispositions to tumor development (Cobrinik et al., 1996).

Determining why *p107* and *p130* can suppress aspects of transformation *in vitro* but inactivating mutations in these genes have not been associated with human or murine cancer remains a key question in the *RB* field. Perhaps one explanation for this apparent paradox could be that the *in vitro* assays are based on overexpressing the proteins. Such overexpression could lead to these proteins simply acting as a surrogate for pRB by binding to E2F complexes which normally only bind to pRB. This could result in the inhibition of these complexes and lead to growth arrest. While this scenario remains possible, the fact that coexpression of E2F-1 can overcome suppression by *RB* but not by *p107* is strong evidence against this (Zhu et al., 1993). Undoubtedly the availability of reagents derived from *p107*, *p130*, and *Rb*-deficient mice will allow a better understanding of the functions of these proteins in the future.

Regulation of pRB phosphorylation

The cellular machinery involved in regulating the phosphorylation status of pRB (as well as p107 and p130) has become well understood in the last several years (see Figure 2-3). Cell cycle progression is controlled by the regulated activity of cyclin-dependent kinases (cdks) (Morgan, 1995). Cyclin dependent kinases were originally identified in yeast and amphibians and subsequently found to comprise a multigene family in mammals (Meyerson et al., 1992; Morgan, 1995). Three of these (cdk2, cdk4, and cdk6) have been recognized to play important roles in progression through the G1 phase of the cell cycle (Sherr, 1994). The catalytic activity of these serine-threonine kinases is positively regulated by their association with cyclin proteins, a family of which at least 7 different subfamilies have been recognized in mammals based on structural homology and function (Hunter and Pines, 1994; Sherr, 1993; Sherr, 1994). In terms of progression from G1 to S phase, D type cyclins (via their association with cdk4 and cdk6) and Cyclin E (through its association with cdk2) play important roles (Draetta, 1994; Sherr, 1993; Sherr, 1994). Both of these cyclin/cdk complexes phosphorylate and presumably inactivate *RB* function (Draetta, 1994; Hatakeyama et al., 1994; Hunter and Pines, 1994; Sherr, 1993; Sherr, 1994) and overexpression of both Cyclins D and E can abrogate the block in cell cycle progression induced by *RB* (Hinds et al., 1992). Cyclins A and B are thought to play important roles in the progression of cells through S and G2/M (Pines, 1995). Cyclin H is a component of the cdk-activating kinase complex (Fisher and Morgan, 1994; Makala et al., 1994; Matsuoka et al., 1994), while the functions of Cyclins C and G have not been determined (Pines, 1995).

Recent data suggests that the only role of D type cyclins (of which three have been identified) in cell cycle control may be in regulating the phosphorylation status of pRB. An elegant set of experiments by Jiri Bartek and colleagues has shown that microinjection of antibodies which inactivate D type cyclins inhibits cell cycle progression only in cells which contain functional retinoblastoma protein (Lukas et al., 1995).

The importance of these regulatory proteins (as well as the pRB pathway in general) is emphasized by the fact that alterations of cdk and/or cyclin expression by chromosomal translocation, gene amplification, or mutation has been observed in many human tumors (Adelaide et al., 1995; Arnold et al., 1992; Motokura and Arnold, 1993; Motokura et al., 1991; Rosenberg et al., 1991; Wolfel et al., 1995; Zukerberg et al., 1995). In addition, analysis of mice deficient for specific D type cyclins are beginning to offer new insights into *RB* function. For example, mice deficient in cyclin D1 exhibit reduced perinatal size and viability, retinal abnormalities associated with decreased retinal size, and a failure to induce proliferation in the breast epithelium in response to pregnancy (Sicinsky et al., 1995). It is tempting to speculate that a failure to inhibit the activity of pRb in these cell types depends specifically on the presence of cyclin D1 (but not D2 or D3). Further evidence suggesting a role for D type cyclins in development and tumorigenesis come from observations of transgenic mice overexpressing Cyclin D1 in the breast (Wang et al., 1994). These mice are predisposed to the development of mammary adenocarcinomas. In addition, coexpression of cyclin D1 and myc in lymphoid tissue can induce lymphomagenesis (Bodrug et al., 1994; Lovec et al., 1994).

The kinase activity of these cyclin/cdk complexes is itself a target of complex regulation. Besides their required association with cyclins for kinase activity, cdks also require the phosphorylation of an internal threonine residue (Thr160 in CDK2) for activity (Solomon, 1994). The phosphorylation of these residues in these cdks is controlled the cyclin/cdk activating kinase (CAK) (Solomon, 1994). CAK was found to be a heterodimer of yet another cyclin/cdk pair (cdk7 and Cyclin H) (Fisher and Morgan, 1994; Makala et al., 1994; Matsuoka et al., 1994). Furthermore, the activity of the CAK is partially controlled by the activity of a yet unidentified kinase. In addition to this level of regulation, cdk activation also requires the removal of inhibitory phosphates from Thr14 and Tyr15 by cdc25 phosphatases (Hoffman and Karsenti, 1994).

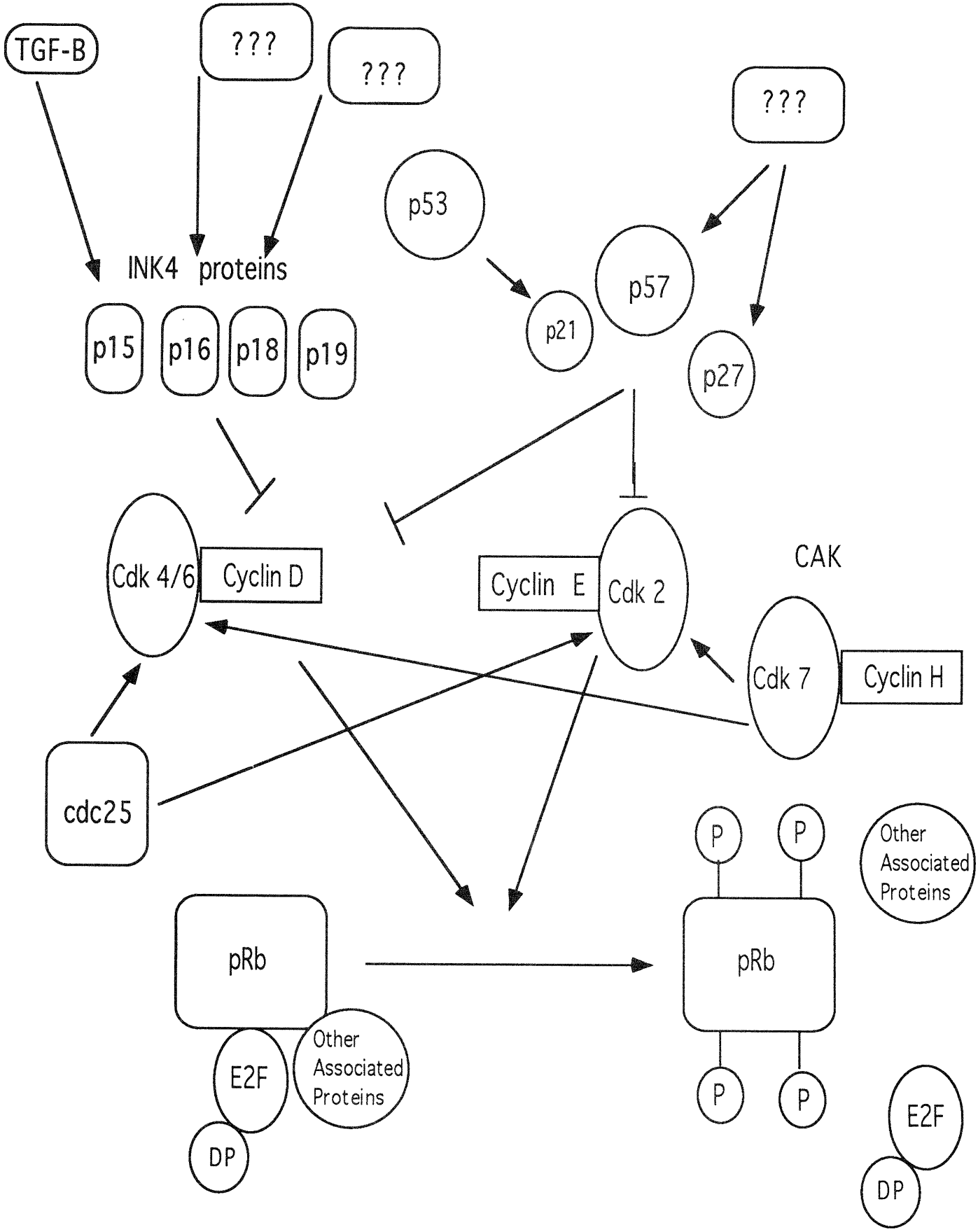
The activities of cyclin/cdk complexes are also controlled by their association with so-called cyclin-dependent kinase inhibitors (CDI). The *WAF1/CIP1/SDI1/p21* is a CDI encoding gene (El-Deiry

et al., 1993; Harper et al., 1993; Noda et al., 1994; Xiong et al., 1993) activated at the transcriptional level by the *p53* tumor suppressor gene protein (El-Deiry et al., 1993). Its activation leads to its association with cyclin/cdk kinases and their subsequent inactivation. Two other genes closely related to *p21* (but not transcriptionally induced by p53), *p27* (Polyak et al., 1994; Toyoshima and Hunter, 1994) and *p57* (Lee et al., 1995b; Matsuoka et al., 1995), have also been recently identified.

The INK4 (Inhibitors of cdk4 kinase) family of proteins are CDIs which specifically inhibit the kinase activity of cyclin D associated cdks. The family presently includes *p15*, *p16*, *p18*, and *p19* (Chan et al., 1995; Guan et al., 1994; Hannon and Beach, 1994; Hirai et al., 1995). The best studied member of this family is *p16*, a gene known to be mutated in a wide variety of cancers, and has been shown to be a strong inhibitor of cell cycle progression through G1. The p16 locus has also been shown to be very complex due to the fact that an unrelated growth suppressing gene ($p19^{ARF1}$) is also transcribed from the same locus (Quelle et al., 1995). Further experiments on p16 have suggested that its ability to block cell cycle progression is dependent on the presence of pRB in the cell (Lukas et al., 1995; Medema et al., 1995). p18 also appears to act through pRB to induce cell cycle arrest (Guan et al., 1994; Hirai et al., 1995).

To a large extent studies on *RB* function have been performed in tumor cell lines known to contain or lack *RB* function (for example SAOS-2 and U2OS cells). While these experiments are valuable, they are often difficult to interpret since tumor cells undoubtedly have undergone a number of genetic changes in order to become transformed and adapted for tissue culture growth. In addition, very little was known about the normal physiological role of Rb during differentiation and development. The ability to create mice with targeted mutations has revolutionized the field of molecular biology, and in this specific case allowed a better understanding of the normal role of *Rb* in development and tumor suppression.

Figure 2-3. Regulation of pRB phosphorylation



Methods for creating mice carrying targeted gene inactivations in the germline

Coincident with the developments in understanding the regulation and function of the retinoblastoma protein, techniques for the creation of targeted alterations in the mouse genome were being developed. One of the seminal events in the development of these techniques came in 1981 when two laboratories reported the isolation of a cell population derived from the inner cell mass of blastocysts (Evans and Kaufman, 1981; Martin, 1981). This population could be grown almost indefinitely in culture and when injected into the blastocoel of another blastocyst could contribute to many tissues in the resulting chimeric mice (Bradley et al., 1984). Since these embryonic stem (ES) cells could contribute to the germline of these chimeras, if methods to create specific mutations in these cells could be developed, it would be theoretically possible to create murine strains carrying any desired mutation for subsequent analysis. However, the nature of recombination in mammalian cells made this task difficult. Unlike yeast, in which exogenous DNA vectors preferentially integrate into the most homologous chromosomal locus, integration into the mammalian genome is a more random process (Mansour et al., 1988; Rothstein, 1983; Shortle et al., 1982; Struhl, 1983). Thus given the size of the murine genome, the probability that a targeting vector would integrate into the homologous site was so low that it was not practical or rational to screen the number of independent clones necessary to uncover the rare homologous integrant. Thus, it was obvious that some method to select or at least enrich for the rare homologous recombinants would be necessary. Although *HPRT* was the first successfully targeted gene in ES cells and subsequently in the mouse germline (Doetschman et al., 1987; Doetschman et al., 1988; Hooper et al., 1987; Kuehn et al., 1987; Thomas and Capecchi, 1987; Thompson et al., 1989), the method of selecting against *HPRT* expression in ES cells was not applicable for selecting for targeted inactivations in genes present in two copies in the ES cell genome (*HPRT* is X-linked and most ES lines used are male). In addition, loss of *HPRT* gene function could be selected for under specific conditions in ES cells; a condition not generalizable to most genes.

In 1988, Mario Capecchi and his colleagues reported a method to selectively enrich for homologously targeted ES cells clones which has now become standard in the field. This method, referred to as positive-negative selection (Mansour et al., 1988), is designed to both positively select for ES cell clones with have integrated the targeting vector anywhere in their genome and select against clones into which the targeting vector has randomly integrated into the genome. To do this, the targeting vector is designed to include a neomycin resistance (*neo*) gene flanked by sequences homologous to the gene being targeted. When the vector integrates into the homologous site it provides a method to select for vector insertion (resistance to G418 conferred by the *neo* gene) and is usually designed to inactivate the gene of interest. The targeting vector also contains a copy of the Herpes Simplex Virus thymidine kinase (*HSV-tk*) gene on at least one end of the vector (outside the region of homology) (Mansour et al., 1988). Since a majority of non-homologous recombination events occur via an integration mechanism which results in the incorporation of the ends of the targeting vector into the genome while homologous events occur via a mechanism which incorporates only the regions flanked by homologous sequences, selection against the presence of the *HSV-tk* gene will enrich for the presence of homologous recombinants. This selection is done by adding the nucleoside analog gancyclovir to the culture media. Since *HSV-tk* is more permissive in its substrate requirements than the endogenous *tk* gene, it (but not the endogenous *tk*) will phosphorylate gancyclovir which allows it to be incorporated into the DNA of the cell eventually compromising the cell's viability (Mansour et al., 1988).

Creation of mouse strains with mutations in tumor suppressor genes

Thus, by the end of the 1980s techniques for the introduction of inactivating mutations into the mouse germline had been developed to a point where their use was practical. Since tumor suppressor gene mutations associated with disease were almost always loss of function mutations (Knudson, 1993) and since the gene targeting technology at the time allowed the relatively easy production of mouse strains with inactivating mutations, they were logical targets for such experiments. Besides the construction of strains carrying targeted mutations in Rb and p53 (both

of which will be discussed in detail below), reports of murine strains in almost all other tumor suppressor genes have been reported. A summary of the effects of these mutations on murine tumorigenesis and development is shown in Table 1-2. While some of these strains develop similar diseases to their human counterparts (Apc and p53) (see Tables 1-1 and 2-1), most of them display phenotypes that are quite different. For example, the most extreme example is illustrated by the differences between mice and humans heterozygous for an inactivating mutation in the Wilms tumor gene (Haber and Housman, 1992; Hastie, 1994; Kreidberg et al., 1993). While children who inherit a mutation in the gene are develop Wilms tumor with extremely high penetrance, mice heterozygous for a targeted inactivated mutation do not show any tumor predisposition. More detailed analysis of the differences in targeted gene disruption phenotypes between humans and mice will be needed to further increase the utility of the mice for modeling human disease syndrome and perhaps will shed insights into the molecular functions of these proteins themselves.

Table 2-1. Effects of mutating murine homologs of human tumor suppressor genes

Gene	Murine Heterozygote Phenotype	Murine Homozygous Mutant Phenotype	References
Rb	Pituitary and Thyroid tumors	Embryonic lethality (e13.5-15.5) Defects in erythropoiesis and neurogenesis	This work and (Clarke et al., 1992; Harrison et al., 1995; Hu et al., 1994; Jacks et al., 1992; Lee et al., 1992; Lee et al., 1994)
p53	Sarcomas, Lymphomas, several others	~10% embryonic lethality with exencephaly; Viable animals develop lymphomas, sarcomas, and other tumors	Appendix A and (Armstrong et al., 1995; Donehower et al., 1992; Harvey et al., 1993; Jacks et al., 1994a; Purdie et al., 1994; Tsukada et al., 1994)
Wt-1	No phenotype	Embryonic lethality (e13.5), Defects in pericardial and urogenital development	(Kreidberg et al., 1993)
Apc	Multiple colon polyps (depending on allele)	Early embryonic lethality (e6.5) depending on allele	(Dove et al., 1994; Fodde et al., 1994; Moser et al., 1990)
Nf-1	Pheochromocytoma Myeloid leukemia	Embryonic lethality (e13.5), Defects in heart development	(Brannan et al., 1994; Jacks et al., 1994b)
Nf-2	Sarcomas	Early embryonic lethality (e8.0)	(McClatchey et al., 1996)
p16^{MTS1}	Sarcomas and lymphomas	Sarcomas and lymphomas	(Serrano et al., 1996)
DCC	??	??	??
VHL	??	??	??
BRCA1	No phenotype	Embryonic lethality (e11.5); neuroepithelial defects	(Gowen et al., 1996)
BRCA2	??	??	

References or reviews that describe the functions of these genes and their association with human cancer can be in Table 1-1.

Initial analysis of mice carrying an inactivating mutation in the *Rb* gene

In 1992, several groups reported the use of these techniques to create a mouse with an inactivating mutation of the retinoblastoma susceptibility gene (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The mice used for the experiments described in this thesis were made by Tyler Jacks when he was a post-doctoral fellow in the laboratory of Robert Weinberg (Jacks et al., 1992). This strain and the other two reported strains exhibited very similar phenotypes. Animals constitutionally heterozygous for an *Rb* mutation do not develop retinoblastoma, instead they develop tumors of the intermediate lobe of the pituitary (Harrison et al., 1995; Hu et al., 1994; Jacks et al., 1992). These growth of the pituitary tumors eventually resulted in the death of the mice between 10 and 18 months of age. It was subsequently shown that they are also susceptible to the development of medullary thyroid carcinoma (see Chapter 4). In both of these tumor types, the remaining wild-type allele of *Rb* is absent from the tumor DNA in almost all cases examined. Mice homozygous for the inactivating mutation in *Rb* are not born, instead they die between days 13.5 and 14.5 of mouse gestation (which is around 20 days). Just prior to the time of death the animals appear severely anemic in that they are noticeable paler than control littermates and display significant amounts of edema. By gross examination the liver also appears smaller. Histological examination of the embryos reveal that the liver is indeed smaller and hypocellular. Examination of tissue sections stained with hematoxylin and eosin shows that some aspect of central and nervous system development is also defective since these tissues contain areas of hypocellularity associated with ectopically placed mitotically active cells and small, dark staining pyknotic cells. The presence of pyknotic cells is diagnostic of apoptotic cell death (discussed in more detail below). These patterns of defects are consistent with the previous analysis of *Rb* expression. While Northern blot analysis showed that the gene was expressed at least at low levels in all tissues examined, the

highest levels of expression are seen in the fetal brain and liver at about 12.5 days of gestation (Bernards et al., 1989).

Fetal liver hematopoiesis is defective in *Rb*-deficient embryos

Murine hematopoiesis is characterized by the presence of several distinct stages. The initial production of red blood cells begins around day 6 or 7 of development within the blood islands of the yolk sac (Dzierzak and Medvinsky, 1995; Moore and Metcalf, 1970). At around day 8.5, blood cells derived from these islands begin to enter the primitive embryonic circulatory system (Dzierzak and Medvinsky, 1995; Moore and Metcalf, 1970). These yolk sac-derived erythrocytes are nucleated and form the majority of cells in the circulatory system until day around day 11 (Dzierzak and Medvinsky, 1995; Moore and Metcalf, 1970). At this time the next site of hematopoiesis, the fetal liver, begins to contribute erythrocytes to the circulatory system (Moore and Metcalf, 1970; Tavassoli and Yoffey, 1983). Fetal liver derived erythrocytes are enucleated and much smaller in size relative to yolk sac derived erythrocytes. The fetal liver remains the dominant hematopoietic organ until later in development when the sites of hematopoiesis shift to the bone marrow and spleen (Tavassoli and Yoffey, 1983).

Rb-deficient embryos appear normal up to 12.5 days of development indicating that yolk sac hematopoiesis is occurring efficiently enough to support viability. However, it should be noted that Clarke et al. did note a minor defect in yolk sac lineage of *Rb*-deficient embryos (Clarke et al., 1992). The appearance of pale, edemic embryos later in gestation coincident with the time that fetal liver hematopoiesis becomes predominant led all three groups to undertake a detailed examination of the circulation and fetal liver (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Analysis of peripheral blood from these embryos revealed the persistence of yolk sac derived erythrocytes suggesting that hematopoiesis from the fetal liver was not occurring efficiently enough to make the switch from nucleated (yolk sac-derived) to enucleated (fetal liver-derived) erythrocytes in the circulation.

As previously described, livers from control littermates at 13.5 days were densely packed with cells while livers from mutant embryos were smaller and obviously hypocellular. The underlying cause of the defect was not clear. In an attempt to better define the defect, Jacks et al. tested the ability of liver cells derived from 12.5 day *Rb*-deficient embryos to differentiate *in vitro* (Jacks et al., 1992). The plating of these cells in conditions favoring erythroid differentiation suggested that the *Rb*-deficient livers differed from wild-type livers in this assay. Hemoglobinized colonies derived from *Rb*-deficient liver cells appeared pale in color relative to controls. In addition, an examination of the cells within these colonies revealed that unlike colonies derived from wild-type cells, colonies derived from *Rb*-deficient cells did not produce mature, enucleated erythrocytes with high efficiency. These results were interpreted at the time to indicate that *Rb*-deficient erythroid precursors were intrinsically defective in their ability to reach terminal, end-stage differentiation (Jacks et al., 1992). Subsequent work in our laboratory suggests that this is not the case. Firstly, analysis of chimeras derived from *Rb*-deficient ES cells reveals that *Rb*-deficient cells can indeed differentiate into mature red blood cells which appear normal (data presented in Chapter 5). In addition, the ability of cells from *Rb*-deficient fetal livers to rescue lethally-irradiated adult mice from death (at least in the short term) also shows that *Rb*-deficient cells are capable of differentiating into mature and functional red blood cells (K. Macleod and T. Jacks, unpublished). The apparent inconsistency of these results with the previously published *in vitro* differentiation results could be explained by a defect in the hematopoietic microenvironment of *Rb*-deficient fetal livers. Such a model would suggest that the *Rb*-deficient cells used in the *in vitro* experiment were not intrinsically defective; instead they were defective at the time they were removed from the embryo because they did not receive a necessary environmental signal to allow them to efficiently differentiate *in vitro* or *in vivo*. When these cells are placed in the presence of a microenvironment which is composed at least partially (see Chapter 5) or fully (in the case of lethally-irradiated wild-type mice) of wild-type cells such a signal is provided allowing at least some level of normal differentiation.

Rb-deficient embryos display defects in neurogenesis

The initial analysis of *Rb*-deficient embryos also revealed the presence of defects in the developing nervous system. One possible explanation for the presence of these defects was that they were secondary to the anemia (and subsequent hypoxia) caused by the defect in fetal liver erythropoiesis. The fact that neurological defects could be noted as early as 12.5 days of development, prior to the time in which the fetal liver deficit significantly compromises viability suggested that this was not the case (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Further analysis by Lee et al. confirmed this (Lee et al., 1994). They showed the underlying cause of the neural defect was the inappropriate proliferation of *Rb*-deficient neurons which should have been permanently withdrawn from the cell cycle. During normal cell differentiation in the central nervous system, cell division is restricted to an area adjacent to the ventricular spaces (the ventricular zone). When the cells begin to terminally differentiate they withdraw from the cell cycle and migrate away from the ventricular zone into zones in which further differentiation takes place (intermediate zone). *Rb*-deficient embryos are characterized by the presence of large numbers of mitotically active cells (measured by incorporation of the nucleotide analog BrdU) in the intermediate zone. Furthermore, the hypocellularity in the zones of the central nervous system distal to the ventricular zone can be explained by the fact that the ectopically proliferating cells are eliminated from the organism by the process of programmed cell death or apoptosis. The association of *Rb*-deficiency with apoptotic induction will be a central concept of the work presented so a brief discussion of the process is included.

Apoptosis

While molecular mechanisms underlying cell proliferation and differentiation have long been the focus of detailed study in biological research, the concept that cells have intrinsic mechanisms with which they can induce their own destruction (programmed cell death) has only recently been fully appreciated . The process of programmed cell death (apoptosis) is clearly different from cell death

induced by severe physical trauma (necrosis) (Kerr et al., 1972). In fact, during normal development large numbers of cells are eliminated by this process. The very term apoptosis (derived from the ancient Greek words used to refer to the falling of leaves from trees) was coined to emphasize the normal physiological nature of the process (Kerr et al., 1972). The steps associated with apoptosis were initially analyzed via electron microscopy and are well described (Kerr et al., 1972). The earliest event in the process has been described as the condensation of nuclear chromatin into condensed masses. Chromatin condensation is also associated with cytoplasmic contraction. Plasma membrane integrity is not compromised; however, protrusions off the surface of the cell (blebs) become apparent. These blebs eventually enclose organelles and other components of the cellular cytoplasm which facilitates their phagocytosis by adjacent cells. In the later stages of the process, nuclear disintegration occurs resulting in characteristic nucleosomal ladders which can be detected via agarose gel electrophoresis. Another common method for the detection of apoptosis is based on the fact that cells undergoing the process fragment their DNA. By utilizing an enzyme (DNA terminal transferase) which can incorporate labeled (usually biotinylated) DNA onto the ends of DNA fragments followed by subsequent staining for the incorporation of biotin, cells undergoing the terminal stages of the process can be identified. The basic concept outlined above is the basis for the TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay (Gavrieli et al., 1992).

As previously mentioned, the physiological importance of apoptosis has only recently been appreciated. Much of the information that has established its importance originated from work performed on the nematode worm, *Caenorhabditis elegans* (*C. elegans*). Over 20 years ago, all the cellular divisions and lineage relationships in the development of *C. elegans* were cataloged (Sulston and Horvitz, 1976). This painstaking process revealed that a significant number of cells were eliminated during *C. elegans* development (reviewed in Ellis et al., 1991; Hengartner and Horvitz, 1994b; Horvitz et al., 1994). Subsequent mutagenesis of the organism produced mutants in which these cell deaths no longer occurred as well as mutants in which extensive cell death occurred. The genes mutated in these screens have been identified in recent years and have been

found to have well conserved homologues in mammalian systems (Ellis et al., 1991; Hengartner and Horvitz, 1994b; Horvitz et al., 1994). The incredible conservation of genes regulating the death inducing pathways emphasized the physiological importance of apoptosis. The demonstration that overexpression of Bcl-2, the human homolog of the *C. elegans* death inhibitory gene *ced-9* (Hengartner and Horvitz, 1994a; Vaux et al., 1992), via chromosomal translocation was a relatively common event in B cell lymphomagenesis (reviewed in Reed, 1994) lead to the realization that interference with these important pathways could lead to tumorigenesis. The role of the *p53* gene in the induction of this process will be described in detail in the following chapter.

Rb-deficiency leads to apoptosis in a variety of contexts

The induction of apoptosis in inappropriately proliferating *Rb*-deficient neurons was described above. In addition, the defect in fetal liver hematopoiesis in *Rb*-deficient embryos is also associated with apoptosis. Fetal livers from 13.5 to 14.5 day *Rb*-deficient embryos are characterized by the presence of large numbers of apoptotic cells as judged by both the presence of pyknotic nuclei and TUNEL-positive cells (K. Macleod and T. Jacks, unpublished). The relevant effected cell type(s) still await identification and characterization. Many other situations where *Rb*-deficiency or inhibition of *Rb* function have been associated with the induction of p53 so the discussion of those will be reserved until the following chapter.

Overall goals of this work

The overall goal of this work is to expand the utility of *Rb*-deficient mice to gain a greater understanding of *Rb* function. It is hoped that this will facilitate the development of more effective strategies for the prevention and/or treatment of a large number of human cancers. Within this broad context two major lines of investigation have been pursued in depth. The first, and more central line of investigation deals with trying to understand why mice (and humans) who carry a defective allele of *Rb* are not highly susceptible to a wider range of malignancies. This seems to

run counter to the observation that pRB plays an important cell cycle regulatory role in most cell types examined. In addition, somatic mutations in *RB* have been associated with the development of many tumor types in humans. The body of work presented here addresses this question by showing that in many tumors mutations in other genes can act in concert with *Rb* mutations to induce tumorigenesis. The best example of that is the work presented in Chapter 3 on the cooperative tumorigenesis seen in mice carrying germline mutations in both *Rb* and *p53*.

The second, less central goal of this work, has focused on explaining how *Rb*-deficient embryos can progress to such a relatively advanced stage of development. This again seems inconsistent with the proposed role of pRB as a nearly universal negative regulator of cell cycle progression. Data presented in Appendix C suggests that the presence of the *RB*-related genes *p107* and *p130* may compensate for the loss of *Rb* and allow development to 13.5 days.

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CHAPTER 3

The *p53* tumor suppressor gene

While this work focuses primarily on the role of the *Rb* gene in suppressing murine tumorigenesis, it relies heavily on the use of mice carrying a targeted mutation in the *p53* gene. For this reason, the identification and functions of the *p53* gene will be reviewed.

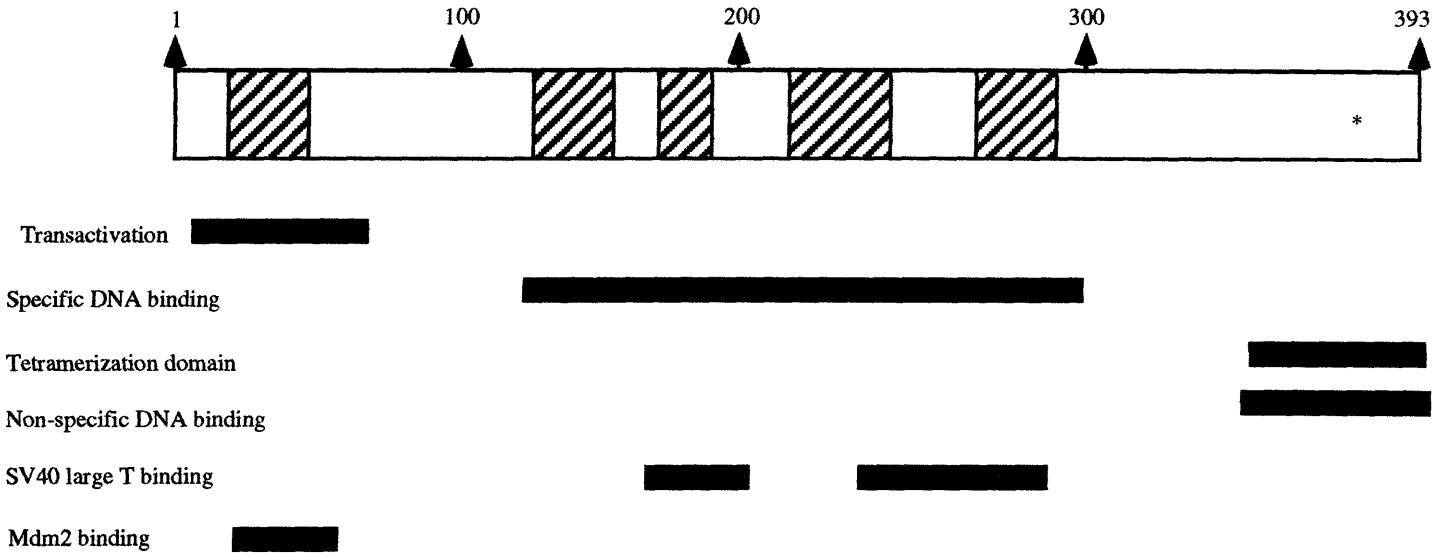
In the seven years since it was recognized to be a tumor suppressor gene, *p53* has been perhaps the most intensely studied gene in the field of oncology. The fact that mutations in *p53* have been estimated to occur in over 50% of all human cancers (Greenblatt et al., 1994; Hollstein et al., 1991) emphasizes the importance of learning more about its functions.

Identification of the p53 protein and gene

In 1979, two groups reported that a 53 kD protein (p53) associated with large T antigen in cells infected with the SV40 polyomavirus (Lane and Crawford, 1979; Linzer and Levine, 1979). Since the protein appeared to share no antigenic determinants with large T antigen and could be detected in cells not infected with SV40 it was thought to be the product of a cellular rather than a viral gene.

The gene encoding p53 was cloned in the early 1980s by several groups independently (reviewed in Levine, 1992). Subsequent analysis showed the gene was located on human chromosome 17p13.1 where it spanned 20 kB, while in mouse it is found on chromosome 11. Eleven exons are present in both humans and mouse, the first of which is untranslated. The gene is extremely well conserved through vertebrate evolution with five regions of the protein showing extensive (90-100%) homology from *Xenopus* to human (Levine, 1992; Levine et al., 1991; Soussi et al., 1987; Soussi et al., 1988) These regions in the corresponding protein product are indicated in Figure 3-1.

Figure 3-1. Characteristics of the p53 protein. The amino acid numbers are noted at the top.



Key

Conserved regions

* Alternative splice (murine-specific)

As expected, the amino acid sequence of p53 is also highly conserved. For example, the human and mouse proteins share 81% homology(Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991; Soussi et al., 1987; Soussi et al., 1988). Analysis of the p53 protein reveals that it has the hallmarks of a transcription factor (see Figure 3-1). The first 75 amino acids of the 393 residue protein can be fused to a heterologous DNA binding domain and stimulate transcription from a heterologous promoter (Fields and Jang, 1990; Raycroft et al., 1990). This region also has the characteristic structure of a well described transcriptional activation domain, the "acidic blob" (Lane and Benchimol, 1990; Levine, 1992; Michalovitz et al., 1991).

Most of the central region of the protein is required for sequence specific DNA binding. The protein has been shown to recognize a consensus nucleotide sequence of TGTCCT(X)$_{5-8}$ TGCCT where X is any nucleotide (Kern et al., 1991). Finally, the C terminus of the protein has been shown to be involved in non-specific DNA binding and required for oligomerization. As previously mentioned, alternative splicing in the mouse, but not human, gene can generate a protein with an altered C terminus (Wu et al., 1993a).

Analysis of mRNA expression suggests that p53 is expressed in all tissues, but with the highest levels found in the spleen and thymus (Reich and Levine, 1984; Schmid et al., 1991). Based on this expression pattern, it was expected that deficiency for p53 would have profound effects on murine development. As discussed below and in Appendix A, this was not the case.

<u>p53 was originally characterized as an oncogene and later as a tumor suppressor gene</u>

Analysis of many transformed cells revealed that p53 levels were elevated relative to normal cells and that more p53 could be detected in rapidly dividing cells relative to arrested cells(Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991). In addition, it was found that the clones of p53 available in the early 1980s could immortalize primary rat cells (Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991) Furthermore,

these clones could also transform these cells in collaboration with oncogenic *ras* and cause primary cells to become independent of platelet-derived growth factor for growth (Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991).

In vivo data also supported the classification of p53 as an oncogene. Introduction of these p53 clones enhanced tumor formation and increased the metastatic potential of tumor cell lines when they were injected into nude mice (Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991).

Some of the first reports which suggested that *p53* may not as an oncogene during tumorigenesis came from studies during the mid 1980s by Benchimol and colleagues who were examining the induction of erythroleukemia by the Friend virus in mice (reviewed in (Johnson and Benchimol, 1992). This work showed that many leukemia clones did not express p53 and this was often associated with rearrangements within the *p53* gene (Chow et al., 1987; Mowat et al., 1985). In 1989 several reports solidly established that *p53* had the characteristics of a tumor suppressor gene rather than an oncogene. It was reported that many of the experiments showing that *p53* could transform primary cells in cooperation with other oncogenes (primarily activated *ras*) had been performed with mutant clones of p53 (Finlay et al., 1989; Hinds et al., 1989; Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991). Transformation assays performed with wild-type versions of the *p53* cDNA showed that it could not cooperate with ras to transform primary cells. The model proposed at the time to explain the earlier results was that some mutant versions of *p53* encoded for proteins possessing dominant negative effects. This model has subsequently received substantial support. p53 is found normally in a homotetrameric complex (Kraiss et al., 1992; Kraiss et al., 1988; Milner and Medcalf, 1991) and some mutated versions of the protein have been shown to alter the activity of the tetramer when cotranslated with wild-type protein (Milner and Medcalf, 1991). Furthermore, the overexpression of p53 observed in many cell lines and tumors is related to the fact that many dominant negative alleles actually increase the stability of the protein. This is presumably due to the fact that such mutations alter the

structure of the protein so it can no longer be recognized by the cellular machinery which normally limit it's half-life (Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991).

Also in 1989, a report on the genetic alterations underlying colorectal tumorigenesis showed that the *p53* gene was commonly mutated in adenomatous and malignant colorectal tissues (Baker et al., 1989). Subsequent examination of a multitude of human tumors has revealed *p53* mutations to be perhaps the most common alteration seen (Greenblatt et al., 1994; Hollstein et al., 1991). For example, about 75% of colon carcinomas (Baker et al., 1989) and 50% of carcinomas of the breast (Baker et al., 1989; Davidoff et al., 1991; Prosser et al., 1990; Varley et al., 1991) and lung (Chiba et al., 1990; Hensel et al., 1991; Iggo et al., 1990; Takahashi et al., 1989) have been shown to contain *p53* mutations. A representative, though not exhaustive list of the cancers in which *p53* mutations have been identified includes cancers of the anus (Crook et al., 1991), bladder (Sidransky et al., 1991), pancreas (Ruggeri et al., 1992), brain (Nigro et al., 1989), esophagus (Hollstein et al., 1990; Hollstein et al., 1991), stomach (Tamura et al., 1991), liver (Bressac et al., 1991; Hsu et al., 1991), ovary (Kohler et al., 1993), uterus (Tsuda and Hirohashi, 1992), skin (Nelson et al., 1994; Ziegler et al., 1994), prostate (Isaacs et al., 1991), and lymphoid system (Ahura et al., 1989; Gaidano et al., 1991), as well as soft tissue sarcomas and osteosarcomas (Diller et al., 1990; Miller et al., 1990; Mulligan et al., 1990; Stratton et al., 1990).

Inheritance of a *p53* mutation in humans is associated with Li-Fraumeni syndrome

Li-Fraumeni syndrome is characterized by an inherited predisposition to several tumor types including sarcomas as well as carcinomas of the breast and brain (Li et al., 1988). Families who carry such a predisposition present a pedigree in which a dominantly inherited gene results in tumorigenesis at a relatively young age. A molecular analysis of such families revealed that affected individuals had inherited a defective allele of the *p53* gene (Malkin et al., 1990; Srivastava

et al., 1990). These inherited mutations cause the loss of function in the allele and subsequent loss of the remaining wild-type allele is associated with the development of tumors.

p53 is targeted by other DNA tumor virus oncoproteins

The realization that SV40 large T antigen associated with both pRB and p53 lead to the investigation of whether other DNA tumor viral genomes encode proteins capable of associating with p53. These analyses revealed that certain strains of human papillomavirus encoded a version of the E6 protein which can be shown to associate with p53 (Werness et al., 1989). Further analysis has shown that this association leads to a decrease in the stability of p53 by promoting its degradation through the actions of the ubiquitin-dependent proteolytic system (Scheffner et al., 1990). Adenovirus also encodes a protein, the E1B 55 kD, which interacts with p53 and inhibits its function (Sarnow et al., 1982; Yew and Berk, 1992). In addition, the E1B 19 kD protein affects p53 by an indirect mechanism (Debbas and White, 1993).

Cellular functions of *p53*

Shortly after *p53* was identified as a tumor suppressor gene, several groups demonstrated that not only did wild-type *p53* could actually inhibit transformation. Examples of this include studies which showed that the wild-type *p53* gene could suppress transformation induced by the cooperative activities of mutant *p53* alleles and other oncogenes (like activated *ras*) (Lane and Benchimol, 1990; Levine, 1992; Levine et al., 1991; Michalovitz et al., 1991). A great deal of subsequent analysis has suggested that *p53* may regulate key aspects of the cellular response to DNA damage by inducing growth arrest or apoptosis in response to it. Additional data has indicated that *p53* function may play a role in maintaining genomic stability.

The first report linking *p53* function to the induction of apoptosis was published in 1991 (Yonish-Rouach et al., 1991). In this work, a temperature sensitive allele of *p53* was shown to rapidly induce apoptosis at the permissive temperature in the myeloid leukemia cell line M1. Subsequent studies reinforced this concept. For example, reintroduction of wild type *p53* into colon carcinoma cell lines also induces apoptosis (Shaw et al., 1992). In addition, work on the DNA tumor virus oncoproteins which bind to p53 indicate that their function may be to inhibit *p53* function in order to prevent the induction of an apoptotic program in virally infected cells. For example, expression of E1A in several cell types grown under various conditions results in the induction of *p53*-dependent apoptosis, a process inhibited by coexpression of E1B 55 kD (Debbas and White, 1993; Lowe and Ruley, 1993).

Another significant development in our understanding of *p53* function came from the demonstration that p53 protein levels were substantially increased in fibroblasts which had been exposed to ionizing radiation (Kastan et al., 1991). Though a similar observation using UV irradiation was reported in 1984 (Maltzman and Czyzyk, 1984), the rediscovery of it by Kastan et al. in 1991 was a critical development. This same group went on to show that the induction of p53 protein levels in fibroblasts lead to an arrest of the irradiated cells (Kastan et al., 1992).

The creation of mice carrying targeted inactivating mutations in the *p53* gene (described below and in Appendix A) provided a valuable resource of reagents to further test *p53* function. One of the best examples is work on the role of *p53* in apoptotic cell death of T lymphocytes. During development in the thymus, self-reactive T cells undergo programmed cell death in the thymus (Robey and Fowlkes, 1994). This process can also be induced in immature thymocytes (CD4+;CD8+) by a number of treatments including exposure to glucocorticoids (Schwartzman and Cidlowski, 1994) and ionizing radiation (MacDonald and Lees, 1990). In immature thymocytes derived from *p53*-deficient animals apoptotic induction induced by ionizing radiation but not by glucocorticoid treatment is abrogated, while in wild-type thymocytes this apoptotic induction by ionizing radiation is associated with a rapid increase in the accumulation of the p53

protein (Clarke et al., 1993; Lowe et al., 1993). This work not only served to more closely link *p53* function with the induction of apoptosis but together with the work of Kastan illustrated that the induction of *p53* could result in different effects depending on the cell type.

Studies of *p53*-deficient embryonic fibroblasts derived from the knockout mice also played a key role in our understanding of *p53* gene function. Introduction of *E1A* into these fibroblasts leads to the induction of *p53*-dependent apoptosis following growth in low serum, treatment with ionizing radiation, or exposure to chemotherapeutic drugs such as adriamycin, etoposide, and 5-fluorouracil (Lowe et al., 1993). Furthermore, the *p53* genotype of tumors derived from murine embryonic fibroblasts transformed with *E1A* and oncogenic *ras* highly correlates with the induction of an apoptotic program after treatment of the tumors with ionizing radiation or certain chemotherapeutic drugs (Lowe et al., 1994). Other examples which implicate the induction of an apoptotic program with the tumor suppressive function of *p53* are described in this work and in the discussion of Chapter 4.

While the idea that the role of *p53* in the induction of apoptosis could explain many aspects of its tumor suppressive functions is generally accepted, another model for the role of *p53* in tumor suppression also has solid experimental support. Importantly, the absence of *p53* has been correlated with genomic instability in established cell lines and in murine embryonic fibroblasts (MEFs) derived from *p53*-deficient mice or fibroblasts derived from Li-Fraumeni patients (Livingstone et al., 1992; Yin et al., 1992). In addition, *p53*-deficient MEFs have recently been recently reported to display abnormal centrosome amplification (Fukasawa et al., 1996). This abnormality significantly effects mitotic fidelity and is consistent with an important role for *p53* in maintaining genomic integrity.

Experiments in animal models have also supported a role for *p53* in maintaining genomic integrity. Transgenic mice in which expression of the *Wnt-1* oncogene is directed to the mammary by the influence of the mouse mammary tumor virus LTRs develop tumors in the mammary

(Tsukamoto et al., 1988). When these mice were intercrossed with *p53*-deficient mice, analysis of *Wnt-1* induced mammary tumors in *p53*-deficient versus wild-type mice revealed that *p53*-deficient tumors exhibited less fibrotic tissue and occurred with a reduced latency (Donehower et al., 1995). More importantly, analysis of DNA derived from these tumors by comparative genomic hybridization indicated that *p53*-deficient tumors contained significantly increased levels of chromosomal abnormalities relative to wild-type tumors (Donehower et al., 1995).

Creation of mouse strains with a targeted disruption in *p53*

As described above, reagents derived from *p53*-deficient mice have proven to be valuable reagents for the study of *p53* function. Analysis of the mice themselves has also provided valuable information about the physiological roles of *p53*. In 1992 Donehower et al. reported the surprising observation that mice deficient for *p53* were developmentally normal but susceptible to the development of several tumor types (predominantly thymic lymphomas and sarcomas during the first six months of life (Donehower et al., 1992). Subsequently three other groups (including our laboratory in work described in Appendix A) reported similar results (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994; Tsukada et al., 1994). It should be noted that further work performed by our laboratory (Appendix A) and confirmed by others has shown that a small but significant percentage of *p53*-deficient embryos develop and succumb to exencephaly (Armstrong et al., 1995; Sah et al., 1995). This suggests that *p53* may play a role in normal embryonic development. Even better evidence for a developmental role comes from studies of mice which contain a targeted disruption in the *p53* target gene *Mdm2* (described below).

The molecular mechanisms of *p53* function

Several lines of evidence suggest that the role of *p53* as a species specific transcription factor underlies its tumor suppressive capacities. Reported transcriptional targets for p53 include the genes encoding for the cyclin dependent kinase inhibitor *p21* (El-Deiry et al., 1993), *mdm2* (Barak

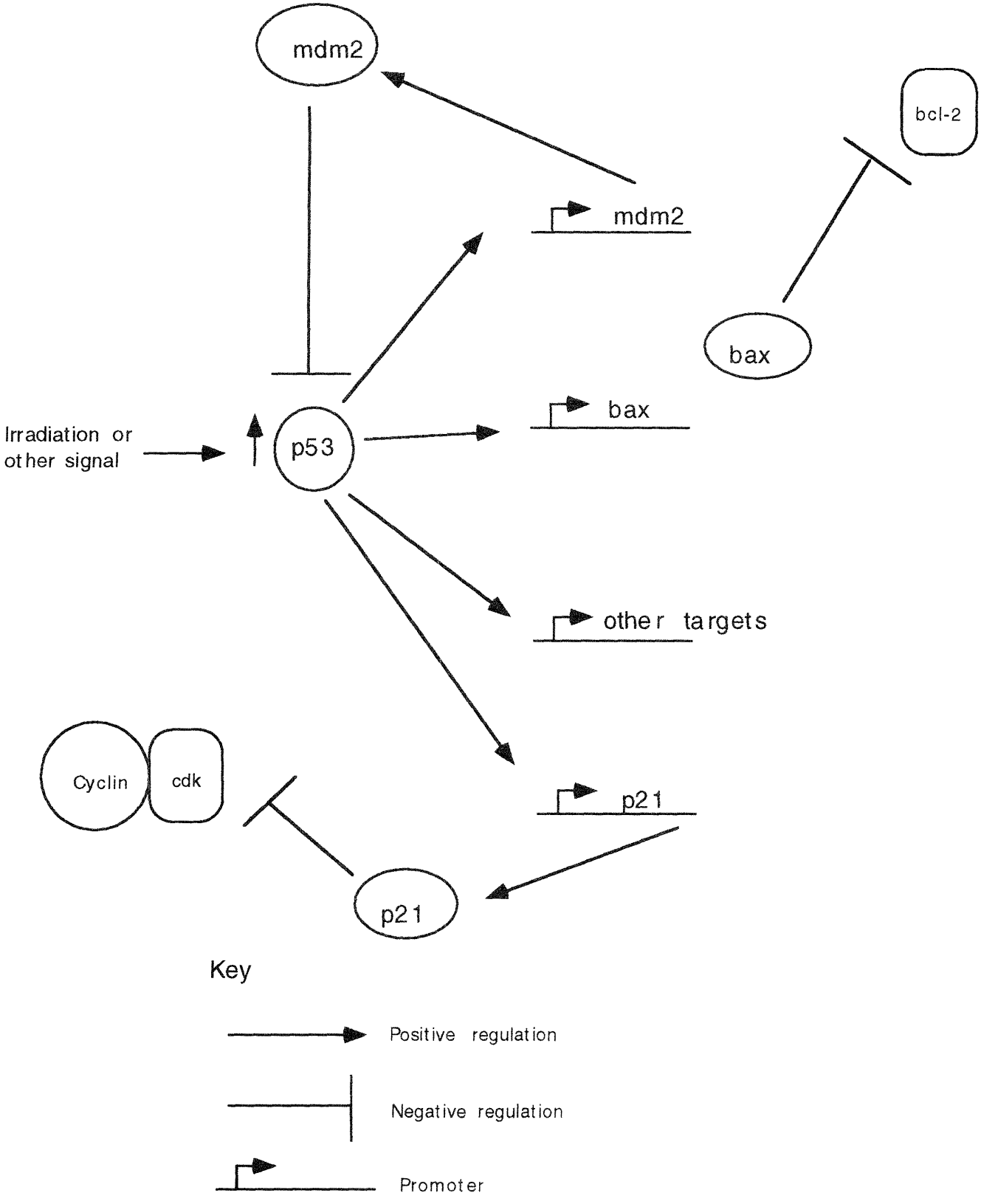
et al., 1993; Perry et al., 1993; Wu et al., 1993b), *gadd45* (Kastan et al., 1992), cyclin G (Okamoto and Beach, 1994), *bax* (Miyashita and Reed, 1995), IGF-binding protein (Buckbinder et al., 1995), and muscle creatine kinase (Weintraub et al., 1991). The implications of *p21* and *mdm2* induction is discussed below. The potential *p53*-dependent induction of *bax*, a cell death promoter via its inhibitory actions on bcl-2 (Oltvai et al., 1993) , and Gadd45 (Kastan et al., 1992) also have important implications for p53 function. Additional evidence which suggests that p53 acts as a transcription factor to suppress tumorigenesis include the fact that many of the mutations seen in human tumors abrogate the specific DNA binding ability of p53 (Ko and Prives, 1996).

Recently the question of whether the transcriptional activity of p53 is necessary for its tumor suppressing functions has been the subject of great debate. Three studies reported recently have suggested that transcriptional transactivation by p53 is not necessary for *p53*-dependent apoptosis to occur. Two of these studies rely on the use of transcriptional and translational inhibitors and show that p53 retains the ability to induce apoptosis in their presence (Caelles et al., 1994; Wagner et al., 1994). The third study reports that p53 mutants which have lost the ability to transcriptionally transactivate retain the ability to induce apoptosis (Haupt et al., 1995). Another set of studies suggest that p53 transcriptional transactivation is required for apoptotic induction. One study shows that a *p53* clone carrying two point mutations in the transactivation domain which renders it unable to stimulate transcription is also defective in inducing apoptosis (Sabbatini et al., 1995). In another study microinjection of different *p53* mutants into *p53*-deficient cells has shown a strong correlation between the presence of a functional transactivation domain and the ability to induce apoptosis (Attardi et al., 1996). Sorting out these conflicting reports will be of great importance in the future.

As discussed in Chapter 2, p21 (also referred to as WAF1, SDI1, and CIP1) has been shown to inhibit the kinase activities of most cyclin dependent kinases. Despite the suggestion that p21 is a major downstream target of p53, only in human prostate tumors have *p21* mutations been detected (Gao et al., 1995). Furthermore, *p21*-deficient mice appear normal and no tumor predisposition

has been noted to date (Brugarolas et al., 1995; Deng et al., 1995). Interestingly, MEFs derived from *p21*-deficient embryos show a partial defect (relative to *p53*-deficient MEFs) in inducing G1 arrest in response to ionizing radiation (Brugarolas et al., 1995; Deng et al., 1995). This suggests that p21 may be an important effector of *p53* function, but that other p53 targets or activities are important in p53 responses (see model in Figure 3-2).

Figure 3-2. *p53* pathway



The *mdm2* gene is transcriptionally transactivated by p53 and the resulting protein product has been shown to associate with and potentially inactivate p53 function. This has lead to the model that mdm2 is a major component of a negative feedback loop which serves to inactivate p53 function (Figure 3-2). This model has received extremely solid experimental support from experiments in knockout mice. Deficiency for *Mdm2* results in early embryonic lethality(Jones et al., 1995; Montes de Oca Luna et al., 1995). When mice deficient for both *Mdm2* and *p53* are created they complete development and are normal fertile adults save for the fact that they develop the same tumor spectrum seen in *p53*-deficient mice (Jones et al., 1995; Montes de Oca Luna et al., 1995). This suggests that p53 overactivity is not compatible with development and that the only role of mdm2 may be to act in a p53 negative feedback loop.

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CHAPTER 4

Cooperative Tumorigenesis Between *Rb* And *p53* Mutations

One explanation for the lack of widespread tumorigenesis in the *Rb* heterozygotes was that while heterozygosity followed by subsequent loss of the wild type allele might predispose many tissues to tumorigenesis, loss of only *Rb* function was insufficient for tumor induction in most tissues. That is, other genetic events may have to occur in combination with *Rb*-deficiency in order to observe tumor development in some tissues. This is is consistent with observations that the development of most tumors is the result of multiple genetic changes ("multi-hit" carcinogenesis) (Vogelstein and Kinzler, 1993; Weinberg, 1989). One obvious candidate for a genetic event that could cooperate with RB mutation to induce tumorigenesis was the inactivation of the *p53* tumor suppressor gene.

The rationale for expecting loss of *Rb* and *p53* function to cooperate in tumorigenesis came from several sources. The first line of evidence is based on the fact that many human tumors have been shown to be associated with mutations in both *RB* and *p53*. These tumor types include sarcomas and carcinomas of the lung, breast, cervix, and pancreas (see below). Perhaps the best example of the concomitant loss of function being implicated in tumorigenesis comes from analysis of cervical carcinoma tumor cell lines (Scheffner et al., 1991). Human papillomaviruses can be detected in approximately 85% of human cervical cancers. The expression of the HPV proteins E6 and E7 has been shown to result in the functional inactivation of p53 and pRB respectively. Thus, no selection for mutations in either *RB* or *p53* should occur during the process of tumorigenesis if HPV is present. Indeed, all five HPV positive cervical carcinoma cell lines (HeLa, C4-II, SiHa, CaSki, and Me180) examined had wild type *RB* and *p53*. In contrast, both HPV negative cell lines examined (C33-A and HT-3) contained mutated *p53* and *RB* genes.

The second line of evidence to suggest that mutations in both RB and p53 could act synergistically in tumorigenesis was inferred from the studies on DNA tumor viruses (papillomavirus, adenovirus, and polyomavirus) described in detail in the previous chapters. In all three cases the activities of the oncoprotein(s) which bind to pRB and p53 are necessary for efficient viral replication.

Finally, several *in vitro* experiments support this idea. For example, several studies have shown that overexpression of the E2F-1 protein can result in p53-dependent apoptosis (Melillo et al., 1994; Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). This overexpression of E2F-1 is presumably equivalent to the loss of RB function since this would also lead to excess free E2F-1 associated complexes. In addition, introduction of the E1A gene into certain cell types can induce p53-dependent apoptosis under certain conditions (Debbas and White, 1993; Lowe et al., 1994b; Lowe and Ruley, 1993).

After the initial analysis of the *Rb* and *p53* mutant mouse strains was completed, the possibility that mutations in *Rb* and *p53* could cooperate in tumorigenesis was tested by intercrossing the strains of mice to generate animals carrying mutations in both genes. The generation of these mice revealed that such cooperative tumorigenesis did exist and the report follows

Cooperative tumorigenic effects of germline mutations in *Rb* and *p53*

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Cooperative tumorigenic effects of germline mutations in *Rb* and *p53*

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The tumour suppressor genes *Rb* and *p53* are mutated in several types of human cancer, and many tumour types carry mutations in both genes. To study how these genes normally function, we and others have created mouse strains with *Rb* and *p53* mutations. Here we describe the phenotypic effects of combined germline mutations in these two tumour suppressor genes. Mice mutant for both genes have reduced viability and exhibit novel pathology including pinealoblastomas, islet cell tumours, bronchial epithelial hyperplasia and retinal dysplasia. These data indicate that mutations in *Rb* and *p53* can cooperate in the transformation of certain cell types in the mouse.

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Tumour suppressor gene mutations occur frequently in the development of human cancer[1]. The retinoblastoma susceptibility gene (*RB*) and *p53* are two of the best studied of these genes. In humans, inheritance of a defective allele of *RB* strongly predisposes to retinoblastoma[2–4], a childhood tumour of the eye. The gene is also subject to somatic mutation during the development of osteosarcomas, soft tissue sarcomas, and carcinomas of the breast, lung and bladder[5]. *p53* is mutated at high frequency in the development of many sporadic human cancers[6] and germline mutations in *p53* are associated with Li-Fraumeni syndrome[7,8].

Work from a number of systems has suggested that the coupled inactivation of p53 and pRb may be important for transformation in some instances. For example, several DNA tumour viruses, including SV40, adenovirus and high transforming strains of human papillomavirus encode proteins that bind and presumably inactivate both pRB and p53, and both of these functions are required for efficient transformation by these viral proteins *in vitro* and *in vivo*(ref. 9 and refs within). Also, several human tumour types, including certain sarcomas[2,10–12] and carcinomas of the lung[13–15], breast[16–20], cervix[21,22] and pancreas[23] show a high frequency of mutation of both *p53* and *RB*. While mutations in *RB* and *p53* occur in the development of many human tumours, the mechanism by which these genes normally suppress tumorigenesis is not clear. The *RB* gene product is thought to act by negatively regulating the passage of cells through the G1 phase of the cell cycle[24], while *p53* has been implicated in negative growth regulation[11,25–28], response to DNA damage[29,30] and the induction of cell death[31–35].

To develop systems to study the activity of *Rb* and *p53*, we and others have created mouse strains with targeted disruptions in their murine homologues[36–40]. Germline mutations in *Rb* and *p53* each predispose mice to cancer[37,39–41]. Animals heterozygous for an *Rb* mutation develop intermediate lobe pituitary tumours (ref. 37 and B.O.W., L.R., R.T.B. & T.J., unpublished observations) within the first 9–15 months of life, and medullary carcinoma of the thyroid occurs in approximately three quarters of these animals (B.O.W., L.R., R.T.B. & T.J., unpublished observations). Animals homozygous for an *Rb* mutation die *in utero* between 13.5 and 15.5 days with defects in fetal haematopoiesis and widespread neuronal cell death[36–38]. The tumour incidence in mice heterozygous for a *p53* mutation is approximately 25% at 16 months of age[40,41] with the most common tumour types being sarcoma and lymphoma. In both *Rb* heterozygous and *p53* heterozygous mice, tumorigenesis usually involves the loss of the remaining wild-type allele of *Rb* or *p53*[37,40,41]. Germline homozygosity for a *p53* mutation causes an even more pronounced cancer susceptibility; 90% of *p53*-deficient animals develop one or more tumours by three to six months of age[39–41].

Generation of *Rb*+/–;*p53*+/– and *Rb*+/–;*p53*–/– mice

To investigate possible cooperative effects on tumorigenesis of germline mutations in *Rb* and *p53*, we mated animals heterozygous for the $Rb^{\times 3t}$ mutation[37] with animals homozygous for the $p53^{\Delta}$ mutation[40] to generate a series of animals heterozygous for both mutations (*Rb*+/–;*p53*+/–). These mice were subsequently intercrossed to yield animals heterozygous for the *Rb* mutation and homozygous for the *p53* mutation (*Rb*+/–;*p53*–/–). Both sets of double-mutant mice were

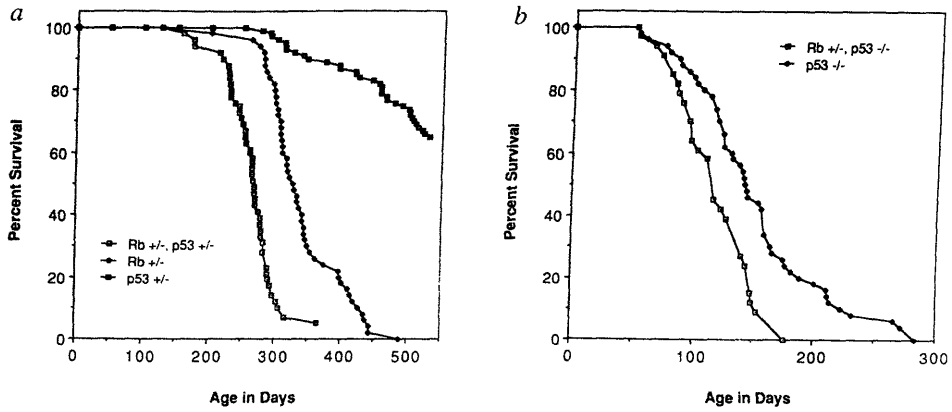


Fig. 1 Survival of mice with mutations in *p53* and *Rb*. *a*, Graph summarizing the viability of 50 *Rb+/–;p53+/–* animals relative to 50 *Rb+/–;p53+/+* and 100 *Rb+/+;p53+/–* animals. The mean age of survival (at which 50% of the animals had died) was 267 days for *Rb+/–;p53+/–* mice and 325 days for *Rb+/–;p53+/+* mice. At 489 days, 75% of *Rb+/+;p53+/–* mice were still alive. *b*, Graph summarizing the viability of 33 *Rb +/–;p53 –/–* animals relative to 50 *Rb+/+; p53 –/–* animals. The mean age of survival was 115 days for *Rb+/–;p53–/–* and 142 days for *Rb+/+;p53–/–*mice.

carefully monitored and animals exhibiting ill health were killed and subjected to complete necropsy.

Analysis of *Rb+/–;p53+/–* mice

The mean age of survival of the *Rb+/–;p53+/–* animals was slightly reduced compared to animals heterozygous for the *Rb* mutation alone (9 months *versus* 11 months) (see Fig. 1*a*). However, the tumour spectrum in these animals was largely the same as that observed in the *Rb* heterozygotes. Each of 67 *Rb+–;p53+/–* animals developed intermediate lobe pituitary tumours and approximately 75% of these mice were also found to have medullary thyroid tumours (Table 1). Additional tumours were detected in seven *Rb+/–;p53+/–* animals: one leiomyosarcoma, three anaplastic sarcomas, two islet cell carcinomas, and one pinealoblastoma. The development of sarcomas was not particularly surprising, as animals heterozygous for *p53* alone are susceptible to this tumour type[40,41], but the three anaplastic sarcomas in the double heterozygotes were more aggressive and arose at an earlier age than those occurring in the single-mutant animals. Tumours of the islet cells of the pancreas or the pineal gland have not been detected in animals with individual mutations in *Rb* or *p53* (Table 1).

We next used Southern blotting to examine the state of the wild-type alleles of *Rb* and *p53* in tumours from *Rb+/–;p53+/–* animals. Previously, we have shown that loss of the wild-type allele of *Rb* occurs during the development of nearly all pituitary and thyroid tumours in *Rb* heterozygotes (ref. 37 and B.O.W., L.R. & T.J., unpublished data), and this was also true in the double heterozygotes. Absence of the DNA fragment associated with the wild-type *Rb* allele was observed in 17/18 pituitary tumours and 6/6 thyroid tumours isolated from these animals (Fig. 2 and data not shown). *p53* mutation occurred rarely in these tumour types, however, as just one of 15 pituitary tumours and one of six thyroid tumours had lost the wild-type allele of *p53* (Fig. 2 and data not shown). Given that the majority of the *Rb+/–;p53+/–* animals developed tumours in which the wild-type allele of *p53* was maintained, we can not readily explain their reduced viability compared to the *Rb* heterozygotes (Fig. 1*a*). In contrast, in each of the six more distinctive tumours that arose in this population of animals (three anaplastic sarcomas, two islet cell tumours and one pinealoblastoma), the wild-type alleles of both *Rb* and *p53* were absent (Fig. 2 and data not shown). Thus, the elimination of function of these two tumour suppressor genes appears to accelerate tumorigenesis in certain tissues in the mouse.

Table 1 Incidence of pathological lesions in mice with different combinations of *Rb* and *p53* mutations

Tumour/lesion	$Rb^{+/-}$	$p53^{+/-}$	$p53^{-/-}$	$Rb^{+/-}$; $p53^{+/-}$	$Rb^{+/-}$; $p53^{-/-}$
Pituitary	100% (204)	0% (44)	0% (56)	100% (67)	94% (16)
Thyroid	70% (27)	0% (3)	0% (8)	83% (12)	60% (15)
Lymphoma	0% (204)	25% (44)	71% (56)	0% (67)	39% (42)
Sarcoma	0% (204)	57% (44)	21% (56)	6% (67)	43% (37)
Islet cell	0% (21)	0% (8)	0% (9)	**14% (14)**	**23% (30)**
Pineal	0% (204)	0% (44)	0% (56)	1% (67)	**40% (42)**
Bronchial hyperplasia	4% (25)	0% (14)	0% (9)	8% (12)	**38% (26)**
Retinal dysplasia	0% (>20)	0% (8)	7% (14)	0% (2)	**41% (17)**

For each type of lesion the incidence is noted, by percentage, with the number of individual animals analysed for that lesion in parentheses. The presence of some tumour types (pituitary, thymic lymphoma, pinealoblastoma and sarcomas) could be assessed by gross necropsy while other lesions (thyroid, islet cell, and bronchial hyperplasia) were detected following histological examination. When a lesion was observed upon necropsy, a piece of tumour tissue was removed for analysis by Southern blotting. Retinal dysplasia was initially diagnosed by ophthalmoscopic examination and subsequently confirmed histologically. Pathological lesions found only in animals with mutations in both *Rb* and *p53* are emphasized by boldface. In addition to the novel tumour types shown, a urethral gland carcinoma (a tumour type not observed in *p53–/–* animals) was also observed in one *Rb+/–; p53–/–* animal. Although thymic lymphoma and several different types of sarcomas are the predominant tumours found in *p53–/–* and *p53+/–* animals, other tumours occur at a lower frequency which have not been included here. Refer to ref. 39–41 for a more complete list of the tumour spectrum of these animals.

Analysis of *Rb+/–;p53–/–* mice

The cooperative effect of germline mutations in *Rb* and *p53* was more apparent from the phenotype of *Rb+/–;p53–/–* animals. The mean age of survival of animals with this genotype was four months (one month less than that of animals homozygous for the *p53* mutation alone), and none of them lived beyond six months of age (Fig. 1*b*). Moreover, the *Rb+/–;p53–/–* animals typically developed numerous distinct tumour foci, with one animal displaying 15 separate lesions affecting eight different tissues. We assume that the reduced viability in this population of animals resulted from their increased tumour burden.

As shown in Table 1, *Rb+/–;p53–/–* animals were susceptible to those tumour types associated with mutation of either *Rb* or *p53* as well as the novel tumours that resulted from the inactivation of both genes noted in the double heterozygotes. Thus, islet cell tumours and

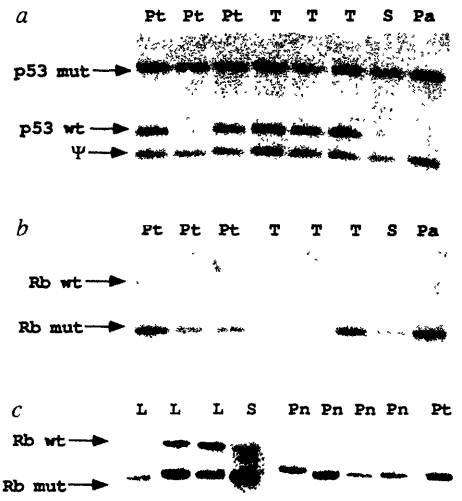


Fig. 2 Status of *Rb* and *p53* in tumours of doubly mutant mice. *a*, Loss of the wild-type allele of *p53* in tumours from *Rb+/-;p53+/-* mice. The wild-type allele of *p53* is absent in the islet cell tumour of the pancreas (Pa), the anaplastic osteosarcoma (S) and one of the pituitary adenocarcinomas (Pt) shown. The wild-type allele is retained in the other two pituitary tumours and in all three thyroid (T) tumours shown. The positions of the wild-type (wt) and mutant (mut) *p53* alleles, as well as the *p53* pseudogene (Ψ) are shown. In total, the wild-type allele of *p53* was absent from 1/15 pituitary adenocarcinomas, 1/6 medullary thyroid carcinomas, 3/3 anaplastic sarcomas, 2/2 islet cell tumours and 1/1 pinealoblastomas examined from *Rb+/-;p53+/-* mice. *b*, Loss of the wild-type *Rb* allele in tumours from *Rb+/-;p53+/-* mice. Representative DNA samples from pituitary adenocarcinoma (Pt), medullary thyroid carcinoma (T), anaplastic osteosarcoma (S) and islet cell tumour of the pancreas (Pa) are shown. In all the tumour types shown the wild-type allele of *Rb* is absent. The positions of the wild-type (wt) and mutant (mut) alleles are indicated. In total, loss of the wild-type *Rb* allele was noted in 17/18 pituitary adenocarcinomas, 6/6 medullary thyroid carcinomas, 3/3 anaplastic sarcomas, 2/2 islet cell tumours, and 1/1 pinealoblastomas examined from *Rb+/-;p53-/-* animals. *c*, Loss of the wild-type *Rb* allele in tumours from *Rb+/-;p53-/-* mice. Representative DNA samples from pituitary adenocarcinoma (Pt), medullary thyroid carcinoma (T), fibrosarcoma (S), pinealoblastoma (Pn) and lymphoma (L) are shown. In the pituitary adenocarcinoma, the fibrosarcoma, one of the three lymphomas and all four of the pinealoblastomas, the wild-type allele of *Rb* is absent or greatly reduced. The positions of the bands corresponding to the wild-type (wt) and mutant (mut) *Rb* alleles are shown. In total, loss of the wild-type *Rb* allele was noted in 3/3 pituitary adenocarcinomas, 1/1 medullary thyroid carcinomas, 7/7 pinealoblastomas, 3/3 islet cell tumours, 3/3 undifferentiated sarcomas and fibrosarcomas, 1/7 thymic lymphomas and 0/4 haemangiosarcomas examined from *Rb+/-;p53-/-* mice.

pinealoblastomas were observed in approximately 20% and 40% of these animals, respectively. In the three islet cell tumours from which DNA was available and in 7/7 pinealoblastomas, loss of the wild-type allele of *Rb* accompanied tumorigenesis (Fig. 2 and data not shown). We also observed three cases of sarcoma in the *Rb+/-;p53-/-* mice in which the wild-type *Rb* allele was lost. In contrast, inactivation of *Rb* was infrequent in development of lymphomas in these animals (1/7 tumours examined), and none of four cases of haemangiosarcoma tested showed loss of the wild-type allele of *Rb* (Fig. 2 and data not shown).

Histological analysis revealed more subtle lesions that were specifically associated with the *Rb+/-;p53-/-* genotype. Approximately 40% of these animals were found to have small foci of basally localized epithelial cells present in bronchi and bronchioles, possibly pulmonary endocrine cells. These regions of bronchial hyperplasia (Fig. 3*c,d*) are histologically similar to some aspects of the recently described human condition, idiopathic diffuse hyperplasia of pulmonary endocrine cells[42]. A direct relationship between these lesions and small cell lung cancer, in which both *Rb* and *p53* are frequently mutated[13–15], has not been established. Also, approximately 40% of the *Rb+/-;p53-/-* mice were found to have pathological changes in the retina (Table 1). However, these focal lesions consisted of areas of retinal dysplasia (or abnormal architecture) as opposed to hyperplasia (see Fig. 3*c*). It is not possible to determine whether these lesions would develop into true retinoblastomas, since the *Rb+/-;p53+/-* animals succumb to other tumours by two to six months of age.

Discussion

The data presented here demonstrate that certain tissues in the mouse are susceptible to transformation upon inactivation of both the *p53* and *Rb* tumour suppressor genes. This provides the first example of cooperativity between two tumour suppressor gene mutations in murine tumorigenesis. These observations are consistent with those made previously in other systems. For example, several DNA tumour viruses encode proteins which bind to and presumably inactivate both p53 and pRB: adenovirus E1A and human papillomavirus (HPV) E7 bind pRb, adenovirus E1B and HPV E6 bind p53, while SV40 large T antigen binds both proteins(reviewed in ref. 9). Also, concomitant mutations of *RB* and *p53* are found in several human tumour types[2,10–23].

We present three models which could account for the cooperative tumorigenic effects of germline mutations in both *Rb* and *p53* (Fig. 4). All three models assume that pRb normally functions as a negative regulator of cell growth, perhaps by inhibiting the activity of bound transcription factors[24], and that *Rb* mutation directly contributes to cellular transformation. The different models address various proposed functions for p53: negative growth regulation[11,25–28], response to DNA damage[29,30] and the induction of cell death[31–35]. In the first model (Fig. 4*a*) *Rb* and *p53* negatively regulate cell growth in a redundant fashion and thus the loss of both is necessary for tumorigenesis in the susceptible tissues. The second model (Fig. 4*b*) is based on the proposed role for *p53* in maintaining genomic stability[29,30,43–45]. Thus, the absence of *p53* might lead to an increased rate of the loss of the wild-type allele of *Rb* in the relevant cell types. Here *p53* mutation indirectly affects tumourigenesis by elevating the overall mutation rate. Our final model (Fig. 4*c*) derives from recent observations that *p53* is required for the apoptotic death of cells expressing the adenovirus *E1A* oncogene[46,47]. Like the expression of E1A (which sequesters pRb (ref. 9) along with other cellular proteins), *Rb* mutation might result in a state of abnormal cell cycle regulation in which cells become prone to p53-dependent apoptosis. Elimination of *p53* function would allow these cells to escape the apoptotic program and progress through tumorigenesis. According to this view, tumour suppression by *p53* is a more active process that rids the animal of cells that have already acquired growth-promoting mutations. Consistent with the third model (Fig. 4*c*), in the context of the developing embryo, absence of *Rb* function does result in widespread cell death[36–38]. Finally, these models are not mutually exclusive and *p53* mutation could

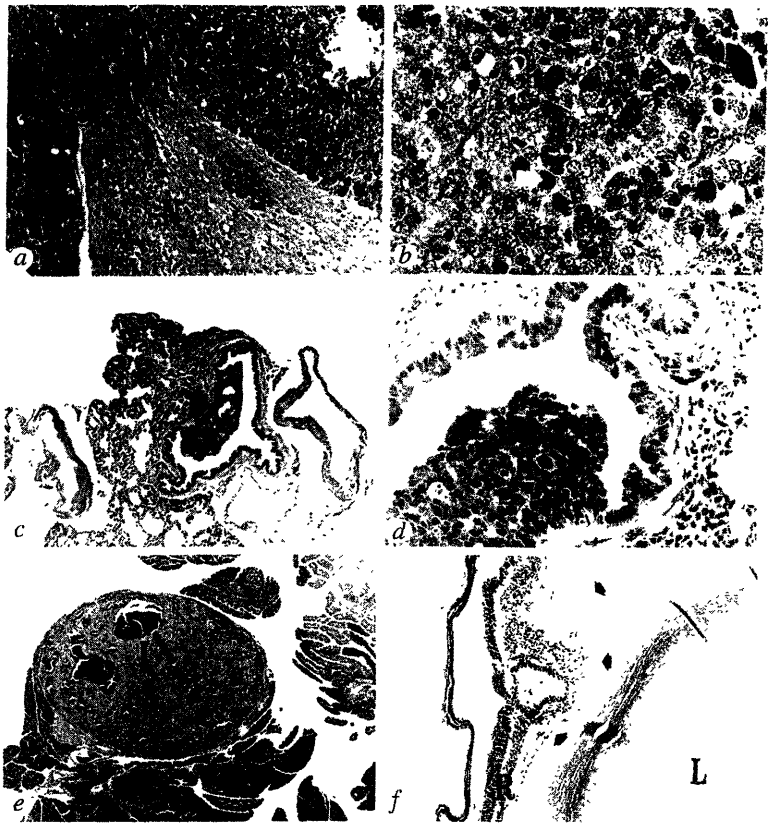
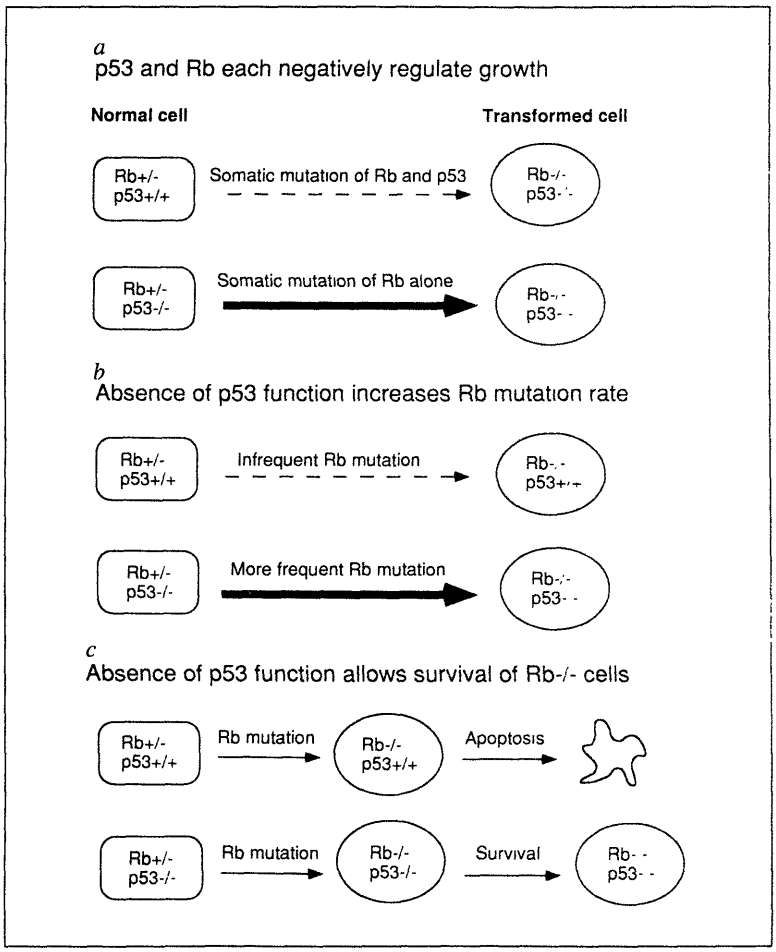


Fig. 3 Novel pathology present in *Rb* +/–; *p53* –/– animals. *a*, Pinealoblastoma (P) growing in normal brain (N). These tumours are highly invasive and aggressive. *b*, Higher power view of a pinealoblastoma showing very pleiomorphic and mitotically active cells (arrow). *c*, Typical hyperplasia of pulmonary epithelial cells. The proliferative cells form a polyp (arrow) projecting into the lumen of a bronchiole. *d*, Higher power view of bronchial hyperplasia (H). Note the transition of normal bronchial epithelium (E) into the hyperplastic region (H). *e*, Islet cell adenoma (I) is shown with normal exocrine pancreas (E) noted. *f*, Area of retina (R) in contact with lens (L). A dysplastic rosette (delineated by arrows) lined by photoreceptor cells is seen overlying the intact pigment epithelial layer. Magnifications: *e*, 40×; *a*, *c*, *d* and *f*, 100×; *b*, 400×.



accelerate tumorigenesis via more than one mechanism.

Of particular interest in this study was the absence of retinoblastoma in mice with germline mutations in both *p53* and *Rb*. Unlike humans[2–4], mice heterozygous for an *Rb* mutation are not predisposed to this tumour type[16–18]. However, retinal expression of the SV40 large T antigen, which complexes with both pRb and p53 (ref. 9), can cause retinoblastoma in the mouse[18,19]. The fact that the *Rb*+/–;*p53*–/– mice did not develop retinoblastoma may suggest that still other functions of SV40 large T antigen (for example, binding to p107 (refs 50, 51)) are required for retinal transformation. Alternatively, the retinal dysplasia observed specifically in the doubly mutant animals might represent a precursor lesion for frank retinoblastoma; the death of these animals due to the development of other tumour types would preclude the further development of these retinal lesions. The fact that the *Rb*+/–;*p53*–/– animals are susceptible to pinealoblastoma is also interesting in this regard, since there are histological and physiological similarities between the pineal gland and the retina[52]. In addition, a small but significant number of familial retinoblastoma patients develop pinealoblastoma, a condition known as trilateral retinoblastoma[53–55].

Finally, it is widely held that most human cancers result from the accumulation of several genetic changes, including the activating mutations in oncogenes and the loss of function mutations in tumour suppressor genes[56]. The data reported here clearly support this "multi-hit" model of carcinogenesis and implicate the mutational inactivation of *p53* and *Rb* as important events in this process. The development of additional mouse strains with germline mutations in other tumour suppressor genes coupled with the large number of transgenic strains expressing dominantly acting oncogenes[57] will allow the systematic evaluation of various mutant combinations and a further dissection of multi-hit carcinogenesis in the whole animal.

Fig. 4 Models for cooperativity between *Rb* and *p53* mutations. Three models are presented which address the cooperative tumorigenic effect of germline mutations in both *Rb* and *p53*. *a*, *p53* and *Rb* function redundantly in negative growth regulation requiring mutational inactivation of both genes. *b*, Absence of *p53* function increases *Rb* mutation rate. *c*, Loss of the *p53*-dependent apoptosis pathway allows *Rb*–/– cells to survive and proliferate. These models are described in more detail in the text. Note that the models are not mutually exclusive, and the observed cooperative effects could result from more than one of them.

Methodology

Genotyping of animals. Animals were genotyped from tail DNA via PCR using three primer systems previously described for the Rb^{x31} (ref. 37) and $p53^{\Delta}$ (ref. 40) mutations.

Southern analysis of tumour DNAs. Tumour samples were surgically removed and DNA was prepared by standard methods. For determination of Rb status, tumour DNA was digested with *Pst*1 and hybridized with a probe derived from the second intron of the *Rb* gene (see ref. 37). *p53* status was analysed by digesting tumour DNA with *Eco*R1 and *Stu*1 and hybridizing with a 3' cDNA probe (probe B in ref. 40). For one islet cell tumour, PCR methods were used to genotype the tumour DNA (see above).

Histological analysis. Tissues were surgically removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm and stained with haematoxylin and eosin prior to microscopic analysis.

Retinal examination by indirect ophthalmoscopy. Pupillary dilation was achieved by topical application of 2.5% phenylephrine HCl and 0.4% tropicamide (1 to 1 dilution with BSS (Massachusetts Eye and Ear Infirmary Formulary)) to each eye. The pupils were fully dilated within 5 min. The retina was then examined using a standard, indirect-ophthalmoscope headpiece in conjuction with 90, 78 or 60 diopter condensing lenses depending on the desired magnification and field. The mouse was not anaesthetized, but was immobilized by an assistant to enable the entire retina to be quickly examined. (S.M., manuscript in preparation).

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The occurrence of novel tumor types in mice carrying mutations in both *Rb* and *p53* proved that mutations in other genes was necessary for tumorigenesis associated with *Rb*-deficiency in many tissues. It will be of interest to see whether mutation in other genes such as the p53 transcriptional target gene *p21* (see Chapter 3) can cooperate with *Rb* mutations in a similar manner.

The incidence of islet cell tumors and pinealoblastoma-like tumors has also been observed in mice expressing SV40 large T antigen specifically in these tissues (reviewed in Van Dyke, 1994). The fact that expression of large T antigen induced tumorigenesis in many tissues not affected in *Rb+/-;p53-/-* mice suggests that other aspects of the multifunctional large T antigen are important in tumor induction in these tissues (for example the choroid plexus) (reviewed in Van Dyke, 1994).

As stated in the discussion of the above reprint, there were several models which could explain the mechanism for this synergy in tumorigenesis. All three models assumed that pRb acts as a transcription factor to inhibit cell proliferation (Hollingsworth et al., 1993; Weinberg, 1995). One model was that both *Rb* and *p53* simply regulated aspects of negative growth control (Baker et al., 1990; Diller et al., 1990; Martinez et al., 1991; Mercer et al., 1990; Michalovitz et al., 1990) and that loss of both was necessary for deregulated growth and proliferation.

A second model for the cooperativity was based on the proposed function of *p53* as the guardian of the genome (Baker et al., 1990; Diller et al., 1990; Lane, 1992; Martinez et al., 1991; Mercer et al., 1990; Michalovitz et al., 1990). The support for this model of *p53* function was outlined in Chapter 2. In this model, *p53* mutation would play a passive role in tumorigenesis by simply increasing the rate at which the wild-type allele of *Rb* is lost. Thus, the loss of *p53* function would not be necessary for tumor formation in these tissues. While we have no evidence that can definitively exclude this model, the fact that none of several hundred *Rb* heterozygous mice created in our laboratory have developed the tumor types seen in the strains carrying mutations in both *Rb* and *p53* argue against this model. If *p53* were only acting to increase the rate at which the wild-

type allele of *Rb* was lost, a small percentage of *Rb*+/- mice would be expected to develop these tumors even when they had two normal genomic copies of *p53*.

The third model we proposed was that elimination of *p53*-dependent apoptosis of *Rb*-deficient cells in these tissue types was the underlying reason for the need to have mutations in both *Rb* and *p53*. By this model, *Rb*-deficiency would simply lead to the *p53*-dependent elimination of *Rb*-deficient cells in these tissues. Only by abrogating this apoptotic program could *Rb*-deficient cells survive and go on to form tumors. This idea was based in large part on the work of Scott Lowe, a graduate student working in our laboratory as well as the laboratories of Earl Ruley and David Housman. Expression of adenovirus E1A in wild-type mouse embryonic fibroblasts (MEFs) resulted in the induction of apoptosis when the cells were grown in low serum or subjected to doses of ionizing radiation or certain chemotherapeutic drugs (Lowe et al., 1993). However, expression of E1A from MEFs derived from *p53*-deficient embryos did not result in apoptosis under these same conditions (Lowe et al., 1993). Since one of the major functions ascribed to E1A is its binding and inactivation of pRB (Whyte et al., 1988), a plausible explanation for the observations was that loss of *Rb* function can lead to *p53*-dependent cell death under certain conditions. We hypothesized that the cooperative tumorigenesis may be based on a similar mechanism; the abrogation of *p53*-dependent apoptosis in certain *Rb*-deficient tissues.

Because we had observed lens defects associated with *Rb*-deficiency (see Chapter 4), we decided to analyze the events of lens differentiation in *Rb*-deficient embryos. To facilitate this, we established a collaborative effort with Sharon Morgenbesser and Ronald DePinho, who had a great deal of experience working with the lens as a system to study developmental events (Serrano et al., 1996). Assays to study the coordination of proliferation, cell death, and differentiation were utilized to examine how *Rb*-deficiency affected lens differentiation. Analysis of lenses from *Rb*-deficient embryos revealed mitotically active lens fiber cells undergoing apoptosis. This cell death was found to be *p53*-dependent when embryos deficient for both *Rb* and *p53* were generated. This work is shown in the following paper.

p53-dependent apoptosis produced by *Rb*-deficiency in the developing mouse lens

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THE retinoblastoma tumour-suppressor gene (*RB*) has been implicated in negative growth regulation, induction of differentiation, and inhibition of cellular transformation[1]. Homozygous inactivation of the *Rb* gene in the mouse leads to mid-gestational lethality with defects in erythropoiesis and neurogenesis[2–4]. Here we describe the effects of the *Rb*-deficient state on the development of the ocular lens. The regional compartmentalization of growth, differentiation and apoptosis in the developing lens provides an ideal system to examine more closely the relationships of these processes *in vivo*. We demonstrate that loss of *Rb* function is associated with unchecked proliferation, impaired expression of differentiation markers, and inappropriate apoptosis in lens fibre cells. In addition, we show that ectopic apoptosis in *Rb*-deficient lenses is dependent on *p53*, because embryos doubly null for *Rb* and *p53* show a nearly complete suppression of this effect. This developmental system provides a framework for understanding the consequences of the frequent mutation of both *RB* and *p53* in human cancer.

In an effort to establish a biological system to study *Rb* function, we and others have used gene targeting to inactivate *Rb* in the mouse[2–4]. Mice heterozygous for the $Rb^{+/-}$ allele (Rb^{+-}) are grossly normal but are highly predisposed to the development of pituitary adenocarcinomas, and inheritance of two mutant *Rb* alleles (Rb^{--}) results in lethality at 13–15 days of gestation[3].

To characterize more precisely the role of *Rb* in the transition from active proliferation to end-stage differentiation as well as to investigate the pathological consequences of alterations in this process, we examined the effects of *Rb* deficiency on ocular lens development[5]. During normal mouse lens development, following lens vesicle formation on embryonic day 11.5 (E11.5), lens cells positioned on the posterior wall of the vesicle withdraw from the cell cycle and elongate in an anterior direction into the vesicle. By E14.5, elongation is complete and results in a lens consisting of postmitotic differentiated fibre cells that are covered anteriorly by a layer of proliferating, immature epithelial cells (Fig. 1*a*). For the purposes of this study, it is important to note that although lens epithelial cells occasionally undergo apoptosis, lens fibre cells do not[6,7] (Fig. 4*a*).

Histological analysis of Rb^{--} lenses from E12.5 E14.5 revealed a significant increase in the number of nuclei as well as occasional mitotic figures in the lens fibre cell compartment (Fig. 1*a*, *b*). That loss of the Rb protein is associated with inappropriate progression through S-phase in lens fibre cells was confirmed by the large number of cells positive for 5-bromo-2′-deoxyuridine (BrdU) throughout the lens fibre cell compartment in Rb^{--} embryos (Fig. 2*b*), but not in wild-type controls (Fig. 2*a*). Given that withdrawal from the cell cycle appears to be a requirement for normal differentiation in many cell types, we examined whether continued proliferation in Rb^{--} lens fibre cells affected their ability to differentiate terminally. Although loss of *Rb* appeared to have a minimal impact on the gross morphological formation of the lens, the highly organized parallel and extended array of normally differentiated lens fibre cells was altered and replaced by a disordered arrangement in *Rb* lenses (Fig. 1*a*, *b*).

As normal lens fibre cell development is dependent upon the proper temporal and spatial expression of α, β and γ-crystallins and the major intrinsic protein of the lens fibre plasma membrane, MIP26 (refs 8, 9), immunofluorescence was used to assay the expression of these stage-specific proteins. Lack of Rb protein did not appear to affect the regional distribution of α-crystallin, an early-stage marker, although the level of staining was mildly reduced in the Rb^{--} lenses relative to wild-type controls (Fig. 3*a*, *b*). In contrast, expression of β- and γ-crystallins and MIP26, the late-stage markers[8–10], was markedly

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decreased in the $Rb^{-/-}$ lens (Fig.3, *c–h*). The more severe reduction in the levels of late-stage markers relative to that of an early-stage marker coupled with the formation of an intact lens structure suggests that the more advanced aspects of differentiation, rather than commitment, are perturbed in the $Rb^{-/-}$ mouse lens.

Previous histological analysis of *Rb*-deficient embryos revealed widespread cell death in the peripheral and central nervous systems[2–4]. Confocal microscopic analysis of propidium-iodide-stained wild-type and $Rb^{-/-}$ lens sections verified the presence of hallmark features of apoptosis, including nuclear fragmentation and chromatin condensation along the nuclear envelope, in many $Rb^{-/-}$ lens fibre cells (data not shown). To establish further that the mechanism of lens fibre cell death was apoptosis, rather than necrosis, we assayed for the presence of excessive DNA 3′-OH ends using the TdT-mediated dUTP-biotin nick-end labelling (TUNEL) method[11]. As anticipated, there was a complete absence of TUNEL-stained nuclei in wild-type lenses (Fig. 4*a*). In contrast, nuclear staining in many lens fibre cells of $Rb^{-/-}$ lenses confirmed the presence of apoptosis in this compartment (Fig. 4*b*) and provided the first clear evidence that the massive cell death that characterizes the *Rb*-deficient phenotype occurs via this programmed mechanism.

Studies in cultured cells have established that *p53* is required for apoptosis associated with deregulated growth[12–15]. However, it is also clear that apoptosis can proceed via *p53*-dependent

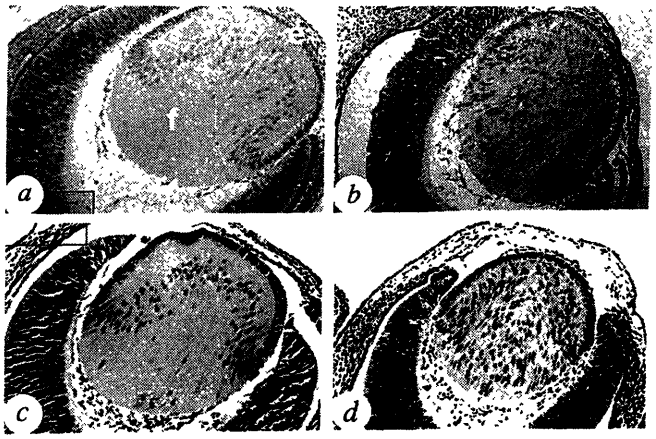

FIG. 1 Morphological development of lenses in E13.5 wild-type and mutant embryos. *a*, Wild-type; *b*, $Rb^{-/-}$; *c*, $p53^{-/-}$; and *d*, $Rb^{-/-}$; $p53^{-/-}$ eye sections. Lenses are oriented with the anterior surface facing the upper right corner. Scale bar, 80 μm. Abbreviations: e, anterior epithelial layer; f, lens fibre cells. The morphology of the $Rb^{-/-}$ lens was indistinguishable from the wild-type (data not shown).
METHODS. Embryos were fixed, sectioned parallel to the optic nerve, and stained with haematoxylin and eosin as described (S.D.M. *et al.*, manuscript submitted). Embryos were genotyped from placental DNAs by PCR as described for the $RB^{\times 3t}$ allele[3] and the $p53^{\Delta}$ null allele[19].

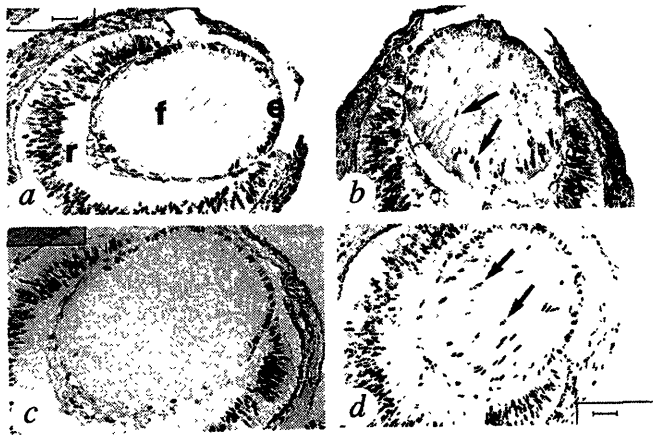

FIG. 2 Lens cell proliferation in E13.5 wild-type and mutant embryos. *a*, Wild-type; *b*, $Rb^{-/-}$; *c*, $p53^{-/-}$; and *d*, $Rb^{-/-}$; $p53^{-/-}$ eye sections. Orientation and scale bar as in Fig. 1. Arrows indicate representative peroxidase-stained, BrdU$^+$ nuclei. Abbreviations as in Fig. 1, and r, retina. Nuclear-associated staining was not observed in the lens fibre cell compartment of $Rb^{-/-}$ embryos (data not shown).
METHODS. Embryos were exposed to BrdU *in utero*, fixed in formalin, embedded in paraffin, sectioned parallel to the optic nerve, and the sections assayed by indirect immunoperoxidase methods using an anti-BrdU/DNA antibody (S.D.M. *et al.*, manuscript submitted). In control experiments, nuclear-associated staining was not detected when the anti-BrdU antibody was not included in the assay (data not shown).

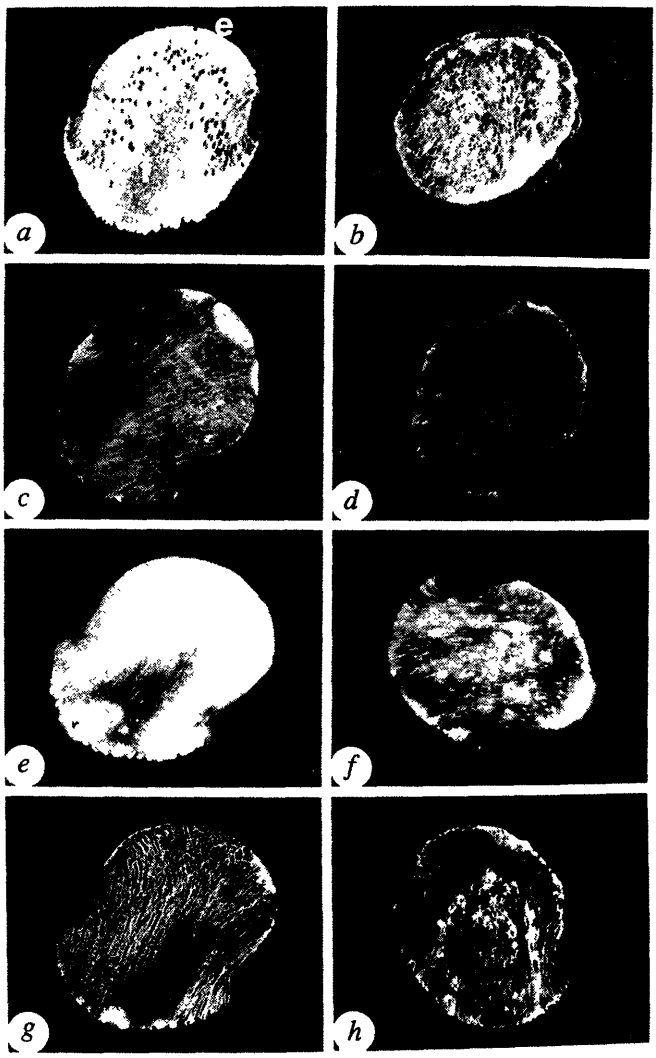

FIG. 3 Cellular differentiation in lenses derived from E14.5 wild-type and mutant embryos. Wild-type (*a, c, e, g*) *and Rb*-deficient (*b, d, f, h*) lenses were assayed for expression of α-(*a, b*), β-(*c, d*) and γ-crystallin (*e, f*), and MIP26 (*g, h*). Orientation and abbreviations as in Fig. 1. Magnification, ×37. In the normal lens, α-crystallin is first observed at the lens vesicle stage and continues to be expressed in both epithelial and fibre cells[8], whereas expression of β- and γ-crystallins and MIP26 begins at E12.5 and is restricted to differentiated lens fibre cells[8,10]. Expression of the crystallins and MIP26 in $Rb^{-/-}$ lenses are indistinguishable from that in wild-type embryos (data not shown).
METHODS. Embryos were fixed, sectioned parallel to the optic nerve, and coronal sections assayed by indirect immunofluorescence with polyclonal antisera against α-, β- and γ-crystallin, and MIP26 (S.D.M. *et al.*, manuscript submitted). Sections stained with the same antibody were photographed with equivalent exposure times. The specificity of these antibodies was confirmed by the absence of staining when the respective preimmune sera were used (data not shown).

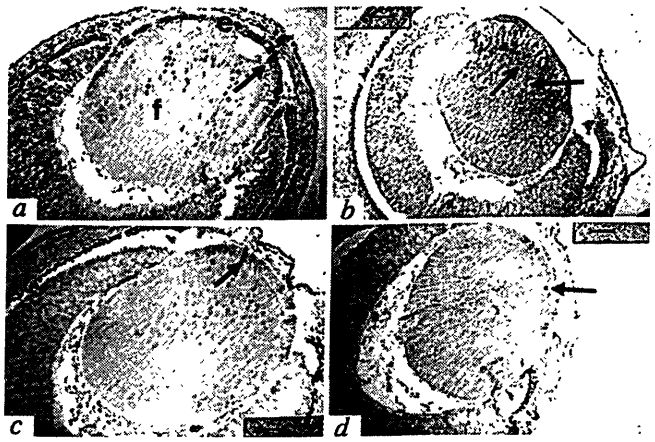

FIG. 4 Apoptotic cell death in lenses from E13.5 wild-type and mutant embryos. a, Wild-type; b, *Rb*$^{-/-}$; c, *p53*$^{-/-}$; and d, *Rb*$^{-/-}$; *p53*$^{-/-}$ eyes. Orientation and scale bar as in Fig. 1. Arrows indicate representative peroxidase-stained, TUNEL^{+} nuclei. The dark rim posterior to the retina is the pigmented layer, and is not due to the extension of free 3′-OH groups by the TUNEL assay. Abbreviations as in Fig. 2. *b* And *d* are representative lens sections of three *Rb*$^{-/-}$ and 3 *Rb*$^{-/-}$; *p53*$^{-/-}$ embryos, respectively. To estimate the number of apoptotic cells per lens section in each embryo, peroxidase-stained nuclei were counted on five sections from the centremost region of the lens and were averaged. *Rb*$^{-/-}$ embryos averaged 12.4, 15.75 and 25.6 apoptotic nuclei per lens section each, whereas *Rb*$^{-/-}$, *p53*$^{-/-}$ embryos averaged 0.6, 1.0 and 1.6. To quantify the reduction in apoptosis in *Rb*$^{-/-}$, *p53*$^{-/-}$ lenses relative to *Rb*$^{-/-}$ lenses, the results for all three *Rb*$^{-/-}$ lenses and for all three *Rb*$^{-/-}$, *p53*$^{-/-}$ lenses were then averaged and compared. For *Rb*$^{-/-}$ and *Rb*$^{-/-}$, *p53*$^{-/-}$ lenses, the mean is 17.92 ± 5.6 and 1.07 ± 0.41 apoptotic nuclei per lens section, respectively, giving a reduction of 94%. Nuclear staining was not observed in the lens fibre cells compartment of *Rb*$^{-/-}$ embryos (data not shown).
METHODS. Embryos were fixed and coronal sections assayed for the presence of DNA free 3′-OH groups using a modified version of the TUNEL assay (S.D.M. *et al.*, submitted). In control experiments, nuclear staining was absent when TdT and biotinylated deoxyuridine were excluded from the assay (data not shown).

or -independent pathways, even in the same cell type[16-18]. To determine whether apoptosis resulting from the lack of *Rb* in the lens was dependent on *p53*, we produced embryos deficient for both of these genes (*Rb*$^{-/-}$; *p53*$^{-/-}$; ref. 19) and examined lens development therein at the most advanced stage possible (E13.5–E14.0). The percentage of TUNEL-stained nuclei in *Rb*$^{-/-}$; *p53*$^{-/-}$ lenses was significantly diminished (by 94%) compared to that observed in age-matched *Rb*$^{-/-}$ embryos (Fig. 4*b*, *d*), indicating that apoptosis in *Rb*-deficient lens fibre cells was *p53*-dependent. The reduction in apoptosis in double-mutant embryos did not correlate with any detectable changes in the rate of lens fibre cell proliferation (Fig. 2*d*), or in the degree of impaired morphological (Fig. 1*d*) and molecular differentiation (data not shown), nor were there any abnormalities in lens development in embryos deficient for *p53* only (Figs 1*c*, 2*c* and 4*c*).

Our results establish a role for *Rb* in the development of the ocular lens. In particular, lens fibre cells lacking *Rb* do not withdraw from the cell cycle before the initiation of end-stage differentiation. These abnormally proliferating cells fail to express normal levels of late-stage differentiation markers and ultimately die by apoptosis. From the analysis of the most advanced lenses from embryos doubly mutant for *Rb* and *p53*, we can conclude that the death of the *Rb*-deficient lens fibre cells is *p53*-dependent. This result is reminiscent of the requirement for *p53* for apoptosis in cells expressing the adenovirus E1A oncoprotein[14,15], which sequesters member of the pRb family as well as other cellular proteins[20,21].

We have also observed abnormal development and extensive apoptosis in lenses of chimaeric mice composed of both *Rb*-deficient and wild-type cells, indicating that this process is lens-cell autonomous[22]. Moreover, we have shown that forced expression of a c-*myc* transgene also leads to deregulated lens fibre cell growth, but, in contrast to the effects noted here, these cells express normal levels of late-stage differentiation markers and do not undergo apoptosis (S.D.M. and R.A.D., manuscript submitted). Therefore, the pathological consequences of *Rb* deficiency may not be accounted for simply by ectopic mitotic activity.

The consequences of null *Rb* and *p53* mutations on lens cell growth and death presented here have important implications for understanding the mechanism through which inactivation of their human counterparts may collaborate in human carcinogenesis. Many tumour types exhibit mutations in both *RB* and *p53* (ref. 19 and references therein) and mice that are *Rb*$^{+/-}$, *p53*$^{+/-}$ develop a wider range of tumours at earlier ages than mice that are either *Rb*$^{+/-}$ or *p53*$^{-/-}$ alone[19], indicating that null mutations in both *Rb* and *p53* can cooperate to bring about malignant transformation, and, that in some cell types, inactivation of both genes may be required. Moreover, the ability of several viruses to transform cells in culture[23-25] and cause tumours in the mouse lens[26,27] requires viral oncoproteins that bind to and inativate both pRb and p53. Due to the attendant lethality of the *Rb*$^{-/-}$; *p53*$^{-/-}$ embryos, we were unable to observe tumorigenic consequences of these two mutations in the developing mouse lens.

Our results show that although loss of *Rb* is sufficient to deregulate lens cell proliferation, this activates a *p53*-dependent programme that leads to the efficient elimination of these abnormally growing cells. This developmental study provides strong corroborating *in vivo* evidence that *p53* may function as a tumour suppressor by promoting the death of pre-malignant cells which could eventually compromise the organism.

Note added in proof: Following submission of this paper, Pan and Griep[28] reported that expression of the pRb-binding protein HPV E7 in the postnatal mouse lens led to effects similar to those described here in the developing *Rb*-deficient lens. Moreover, co-expression of HPV E6 led to a reduction in apoptosis and to lens tumour formation.

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The work described above illustrated that cells lacking *p53* could survive under conditions (*Rb*-deficiency) where normal cells would not. However, because the embryos deficient in both *Rb* and *p53* die shortly after the time this analysis was done (at approximately 13.5 to 14.5 days p.c.), it could not be determined whether this survival would eventually lead to tumorigenesis. However, work from other groups has supported the idea that the inappropriate survival of these cells could result in tumorigenesis. Work from the laboratory of Anne Griep has shown that concomitant expression of HPV E6 and E7 proteins driven by a lens specific promoter leads to the development of lens tumors in the mouse (Pan and Griep, 1994). Expression of only E7 leads to the induction of apoptosis in the lens during development, causing the formation of cataracts in adult transgenic mice (Pan and Griep, 1994). While these experiments are a nice extension of the work presented here, they are complicated by several factors, the most important being that E7 and E6 bind to and affect the function of many other proteins besides pRb and p53. As discussed previously, E7 is able to bind to and presumably inactivate the pRB-related proteins p107 and p130. In addition, recent evidence suggests that it can also bind to and positively affect the activity of AP-1. E6 has been shown to interact with the putative calcium binding protein ERC-55 (Lowe et al., 1994a) and also affect the activity of telomerase in a manner independent of its ability to bind to p53 (Helin et al., 1993). Furthermore, more recent work by the Griep laboratory has shown that while apoptotic cell death induced by E7 (and by extension probably *Rb*-deficiency) is *p53*-dependent before day 13.5, E7-induced lens fiber cell apoptosis at later stages of development is blocked by E6 expression, it is not abrogated by *p53*-deficiency (Pan and Griep, 1995). This illustrates that other functions of E6 play roles in the abrogation of apoptosis. Perhaps the utilization of the aphakia blastocyst complementation assay (Liegeois et al., 1996) with ES cells deficient for different combinations of the pocket proteins and *p53* will help to clarify some of these issues (see Appendix E).

Aside from the lens, studies in other systems have also supported the idea that *p53*-dependent cell death is induced by inhibition of *Rb* (or *Rb* family function) and that abrogation of this apoptotic response can result in tumorigenesis. Transgenic mice expressing HPV E7 specifically in the

retina exhibit widespread apoptotic cell death in the developing retina (Howes et al., 1994). When this same transgene in present in *p53*-deficient mice, retinoblastoma is observed (Howes et al., 1994). Thus, deficiency for both *p53* and *Rb*-family function results in tumorigenesis in this tissue. In this case, however, some evidence suggests that loss of multiple members of the *Rb* gene family is necessary for tumorigenesis as *Rb*+/-;*p53*-/- mice do not develop retinoblastoma. Indeed, mice carrying mutations in both *Rb* and *p107* develop severe retinal dysplasia (see Appendix C). This coupled with the observation of milder retinal dysplasia in *Rb*+/-;*p53*-/- mice may indicate that loss of all three proteins' functions may be necessary to cause murine retinoblastoma.

Perhaps the most direct demonstration of *p53*-induced apoptosis suppressing tumorigenesis associated with Rb-deficiency is reported by Symonds et al. (Symonds et al., 1994). SV40 T antigen expressed specifically in the choroid plexus epithelium induces choroid plexus tumors (Van Dyke, 1993). A truncated form of T antigen which retains the ability to bind *Rb*-family members but lacks the capacity to complex with p53 (TruncT) induces choroid plexus tumors with longer latency when expressed in the same way as wild-type T antigen (Chen et al., 1992). Examination of the choroid plexus epithelium of these animals (TruncT) revealed both excessive mitotic activity and widespread apoptosis (Symonds et al., 1994). The apoptosis, but not the excess proliferation was abrogated when this transgene was expressed in a *p53*-deficient background (Symonds et al., 1994). In addition, tumor latency in the *p53*-deficient background was reduced relative to the tumors occurring in *p53* wild-type animals. In the most revealing observation, animals carrying the TruncT transgene and heterozygous for the *p53* mutation exhibit high levels of proliferation and apoptosis in the choroid plexus epithelium, but exhibit nodular regions of tissue in the choroid plexus epithelium with dramatically reduced amounts of apoptosis. This is associated with a increased rate of tumor growth. In these regions it can be shown that the wild-type allele of *p53* has been lost (Symonds et al., 1994).

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Note: Additional relevant references may be found within this chapter on pages 103 and 108.

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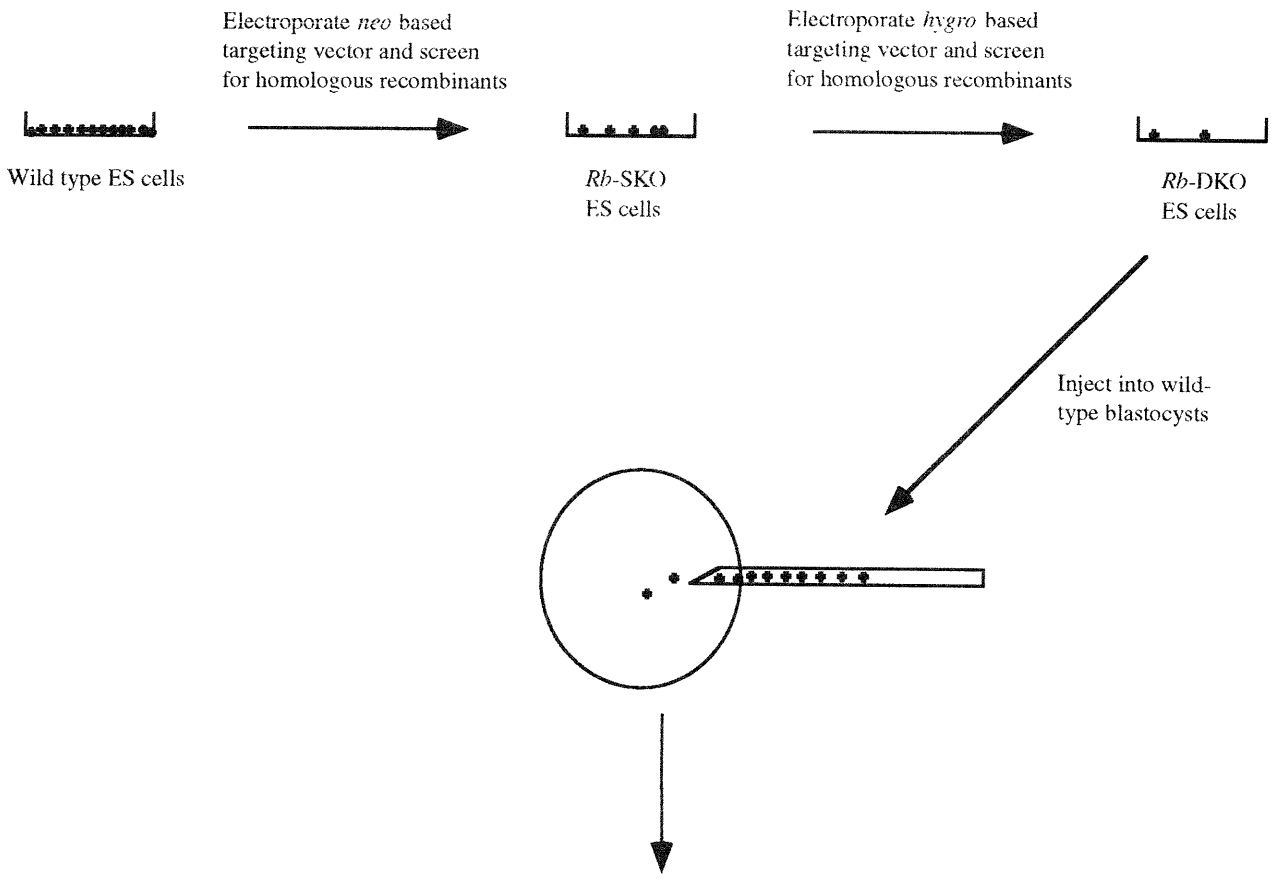
CHAPTER 5

Use of Chimeric Mice to Assess Effects of *Rb*-Deficiency in Adults

While the creation of the *Rb*-deficient mouse strain yielded many insights into the role of *Rb* in murine development and tumorigenesis, the embryonic lethality associated with germline homozygosity for *Rb*-deficiency somewhat limited the strain's utility. In order to overcome this limitation and examine how *Rb*-deficiency would affect later stages of development, we created ES cells with both copies of the *Rb* gene carrying targeted inactivating mutations and injected them into blastocyst to create chimeric mice composed partially of cells derived from the *Rb*-deficient ES cells. We correctly assumed that the presence of wild-type cells in the chimeras would rescue the *Rb*-deficient cells from their normal embryonic lethality in *Rb*-deficient embryos and allow *Rb*-deficient cells to form portions of many adult tissues. Not only did we feel that this would allow a more complete examination of the effects of *Rb*-deficiency on mouse development, but it would also allow us to address one possibility for why *Rb* heterozygotes do not develop more widespread tumorigenesis. This was that loss of the wild-type allele of *Rb* did not occur at a sufficient rate in some tissues (perhaps because of a small target size) to allow tumors to form. By seeding adult tissues with cells already deficient for both copies of *Rb*, we expected to see an enhancement of tumorigenesis.

The chimeric mice analyzed in this experiment were generated from ES cells with both copies of the *Rb* gene disrupted by two successive rounds of gene targeting. A schematic representation of the chimera generation process is shown in Figure 5-1.

Figure 5-1. *Rb*-DKO chimera generation



Analyze chimeric offspring

Because the ES cells are derived from the 129/Sv (129) strain of mice and the blastocysts are derived from the C57/Bl6J (B6) strain, the cells derived from these two different founders in the chimeras contain several polymorphic strain differences. These polymorphisms were exploited to examine these chimeras. The most obvious difference between the two strains is that mice of the 129 strain are agouti while mice of the B6 strain are black (Bradley, 1987). While this is useful for identification of chimeras (and the identification of germline derived offspring from these chimeras), it reveals nothing about the level of chimerism in any other tissue (although one usually assumes a general positive correlation between the level of coat color chimerism and contribution to the internal organs). In this study, we took advantage of two other differences in the strains which were polymorphisms in the *glucose phosphate isomerase (GPI)* locus (Bradley, 1987) and in the hemoglobin locus (Whitney III, 1977). GPI is an enzyme which because of its necessary role in the glycolytic pathway, is present at relatively high concentrations in all living cell types (Bradley, 1987). The 129 and B6 isoforms of the proteins differ enough that they can be electrophoretically separated (Bradley, 1987). By making homogenates of various chimeric tissues, and then running them on a cellulose acetate plate, the two isoforms can be separated and the relative amounts of each isoform in the homogenates can be quantitated. This analysis showed that all adult tissues contained contribution of *Rb*-deficient cells. While this result was interesting, one must keep in mind that the GPI assay measures contribution at the level of tissues. Because we had no way of identifying *Rb*-deficient cells at the cellular level via immunohistochemistry or other methods, the analysis was limited to the tissue level of resolution. This limitation will be addressed in detail in the following chapter.

While the data from the GPI assay demonstrated that *Rb*-deficient cells were present in all tissues examined, it did not address whether this contribution resulting in normal appearing tissue. To examine this question, extensive histological analysis was undertaken. Surprisingly, this analysis showed that only a limited number of tissues (lens, pituitary, cerebellum, adrenal, and liver) exhibited noticeable pathological changes.

In addition to the GPI polymorphism, red blood cells derived from the two mouse strains contain different isoforms of the B-chain of hemoglobin which can be electrophoretically separated in a manner similar to that of GPI (Whitney, 1977). Because of the hematopoietic defect noted in *Rb*-deficient embryos (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), and the limitations of the GPI assay (see below), this assay was used to unambiguously assay for the presence of *Rb*-deficient red blood cells in the adult chimeras. In perhaps the most surprising aspect of these experiments, the presence of significant levels of *Rb*-deficient red blood cells in adult chimeras was confirmed. The report of this work follows.

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Extensive contribution of *Rb*-deficient cells to adult chimeric mice with limited histopathological consequences

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Homozygosity for a mutation in the *Rb* tumor suppressor gene causes mid-gestation embryonic lethality in the mouse. Using a two-step targeting protocol, we have constructed *Rb* homozygous mutant mouse embryonic stem cells and used them to create chimeric animals partially composed of *Rb*-deficient cells. Analysis of these chimeras demonstrates widespread contribution of the mutant cells to adult tissues, including the retina and mature erythrocytes. Despite the presence of large numbers of *Rb*-deficient cells in most tissues of these mice, they are remarkably normal but do exhibit certain histological defects including cataracts, hyperplasia of the adrenal medulla, and enlarged cells in the cerebellum and the liver. Like animals heterozygous for the *Rb* mutation, the chimeras develop tumors of the intermediate lobe of the pituitary, and the rate of pituitary tumorigenesis is greatly accelerated.

Key words: embryonic lethality/mouse/*Rb* tumor suppressor gene/tumorigenesis

Introduction

The development of many human tumors is associated with mutations in tumor suppressor genes (Knudson, 1993). One of the best studied of these genes is the retinoblastoma susceptibility gene (*RB*). In humans, inheritance of a defective allele of the *RB* gene strongly predisposes to pediatric eye cancer, retinoblastoma (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). The gene is also mutated during the development of sporadic retinoblastomas as well as osteosarcomas, soft tissue sarcomas, and carcinomas of the breast, lung and bladder (Friend *et al.*, 1986, 1987; Fung *et al.*, 1987; Lee *et al.*, 1987; Harbour *et al.*, 1988; E.Y.-H.P.Lee *et al.*, 1988; T'Ang *et al.*, 1988; Yokota *et al.*, 1988; Varley *et al.*, 1989; Bookstein *et al.*, 1990b; Hensel *et al.*, 1990). Introduction of wild-type *RB* into various tumor cell lines which have lost endogenous *RB* function can inhibit tumorigenicity and cell cycle progression or induce cell senescence (Huang *et al.*, 1988; Bookstein *et al.*, 1990b; Qin *et al.*, 1992). These results provide direct evidence that this gene can act to limit cell proliferation and that its loss contributes to the decontrolled growth associated with these tumor cell types.

To study the function of *RB* in more detail, we and others have created mouse strains carrying germline mutations in its murine homolog, *Rb* (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Analysis of animals heterozygous for the targeted disruption revealed no evidence of predisposition to retinoblastoma. However, the heterozygous animals were highly susceptible to adenocarcinoma of the pituitary, with ~90% of the animals developing this tumor by 1 year of age (B.O.Williams, L.Remington, R.T.Bronson and T.Jacks, unpublished data). In addition, heterozygotes are susceptible to medullary thyroid carcinoma, with 70% of animals developing this tumor by 1 year of age (B.O.Williams, L.Remington, R.T.Bronson and T.Jacks, unpublished data). As with human retinoblastoma (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987), pituitary and thyroid tumorigenesis in these mice was accompanied by the mutation of the wild-type allele of *Rb* (Jacks *et al.*, 1992; B.O.Williams, L.Remington, R.T.Bronson and T.Jacks, unpublished data).

The *Rb* mutant mice also provided the opportunity to study the role of this tumor suppressor gene in normal development. Homozygosity for the *Rb* mutation was shown to cause embryonic lethality between days 13.5 and 15.5 of gestation (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Death was associated with defects in the production of mature erythrocytes in the liver and widespread cell death in the central nervous system. The development of other tissues, including the retina, appeared to occur normally in the *Rb*-deficient embryos.

The analysis of homozygous mutant embryos has suggested a requirement for *Rb* in normal development of at least erythroid and neuronal tissues. Due to the attendant lethality, however, it was not possible to determine what effects, if any, the absence of *Rb* function would have on normal development or neoplasia at later stages of gestation or postnatally. To address these issues, we have created embryonic stem (ES) cells that are homozygous for the *Rb* mutation and injected them into wild-type blastocysts, yielding chimeric animals. Due to the contribution of the wild-type cells from the host embryo, many of these chimeras escape the mid-gestation lethality caused by germline *Rb* homozygosity and develop to birth. This has allowed us to assess the pathological and tumorigenic effects of the widespread presence of *Rb*-deficient cells in later stages of development and in adult life.

Results

Construction of homozygous mutant ES cells and generation of chimeric animals

Rb homozygous mutant (*Rb*-DKO) cells were created using a two-step targeting protocol (te Riele *et al.*, 1990).

First, heterozygous D3 ES cells (Gossler *et al.*, 1986) (derived from strain 129/sv) were constructed using an *Rb* targeting vector carrying a neomycin resistance gene (*neo*) (Jacks *et al.*, 1992). The surviving wild-type *Rb* allele present in a single heterozygous ES cell clone was then re-targeted using a second vector, identical to the first except that a hygromycin resistance gene (*hygro*) was substituted for the *neo* gene (Figure 1A). *Rb*-DKO cells were identified by Southern blotting following selection in G418, hygromycin and gancyclovir.

As shown in Figure 1B, resulting homozygous mutant cells lack the band corresponding to the wild-type *Rb* allele and contain instead an additional fragment representing the *Rb-hygro* mutation. Of 173 ES cell clones resistant to the three drugs, six were found to be homozygous for the *Rb* mutation, with the herpes simplex virus thymidine kinase (*HSV-TK*) counterselection (Mansour *et al.*, 1988) providing an ~6-fold enrichment for correctly targeted clones. Thus, the efficiency of targeting the second *Rb* allele was approximately one in 173 hygromycin-resistant cell clones, similar to that observed for the first round of gene targeting (Jacks *et al.*, 1992). Consistent with earlier analysis of embryos homozygous for the original Rb^{-} mutation (Jacks *et al.*, 1992), the *Rb*-DKO ES cells were also found to lack detectable *Rb*-encoded protein (pRb) by Western blotting (data not shown).

Three independently derived *Rb*-DKO ES cell clones were then injected into C57Bl/6 blastocysts to create chimeric animals. For comparison, several chimeras were also generated using the parental heterozygous ES cell clone. Given the lethality caused by germline homozygosity for the *Rb* mutation, we assumed that injection of the standard 12–15 ES cells per blastocyst (Bradley, 1987) might lead to embryonic lethality and reduced the number of *Rb*-DKO cells injected per blastocyst to approximately eight. A systematic analysis of the effects of injection of different numbers of mutant cells was not performed.

Contribution of Rb-DKO cells to adult tissues

The contribution of the *Rb*-deficient cells to chimeric animals could be estimated from the extent of agouti coat color (the product of the *Rb*-deficient 129/SV ES cells) and was found to range from ~30 to 60%. In addition, we wished to determine the percent contribution of mutant cells to the internal tissues of these chimeras. The *Rb*-DKO ES cells are homozygous for the *a* allele of glucose phosphate isomerase (*GPI-1s*^{a}, encoding GPI-1A) while cells of the C57Bl/6 host embryo are homozygous for *GPI-1s*^{b} (encoding GPI-1B) (Lyon and Searle, 1990). Thus, by using cellulose acetate electrophoresis and staining for the specific enzyme activity of the two isoforms (Bradley, 1987), we could determine the relative contribution of the *Rb* −/− cells to individual tissues (Figure 2A). More than 35 tissues from 14 *Rb*-DKO chimeras and five controls were examined using this assay.

As summarized in Figure 2B, we observed significant contribution of the *Rb*-DKO cells to all adult tissues examined, including liver, thymus, spleen, kidney and muscle. These data indicate that *Rb* function is not required for the differentiation of cells in many adult tissues. However, since the GPI assay only measures the percent contribution to the tissue as a whole, it is not possible to

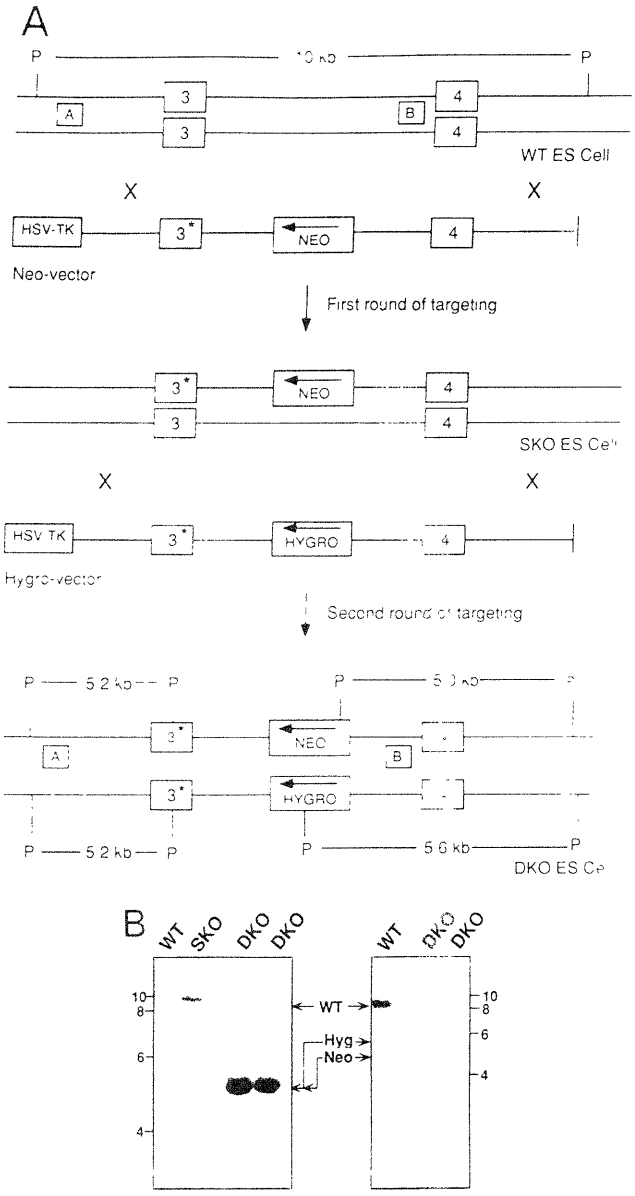

Fig. 1. Construction of *Rb*-deficient cells. (**A**) Gene targeting strategy to create *Rb*-DKO ES cells. One allele of the *Rb* gene was mutated in wild-type (WT) D3 ES cells (Gossler *et al.*, 1986) in the first round of targeting using a vector carrying a mutation in the third exon (designated 3*) and a neomycin resistance gene (*neo*) in the third intron. The exon 3 mutation creates two tandem termination codons and a novel *Pst*I (P) recognition site (Jacks *et al.*, 1992). The remaining wild-type allele in the heterozygous (or SKO) ES cell was then subjected to a second round of gene targeting with a vector identical to the first except that the hygromycin resistance gene (*hygro*) replaced *neo* (*hygro* targeting vector), yielding the *Rb*-deficient (or DKO) ES cells. Both targeting vectors contain the herpes simplex virus thymidine kinase gene (*HSV-TK*) to provide counterselection (Mansour *et al.*, 1988). As indicated by arrows, the drug selection genes are in the opposite transcriptional orientation from *Rb*. The lengths of homology to *Rb* in both targeting vectors are 1.1 kb upstream of the drug resistance gene and 8.5 kb downstream (Jacks *et al.*, 1992). Clones were screened by Southern blotting of *Pst*I-digested genomic DNA using probes A and B (boxed). Lengths of relevant *Pst*I restriction fragments are indicated. (**B**) Southern blots of *Rb*-mutant ES cells. *Pst*I-digested genomic DNA isolated from wild-type ES cells, the parental SKO ES cell clone and several DKO ES cell clones was hybridized to probes A (left) and B (right) shown in panel A. Positions of the wild-type (WT) *neo* and *hygro* mutant alleles are shown along with the positions of molecular weight standards (in kb).

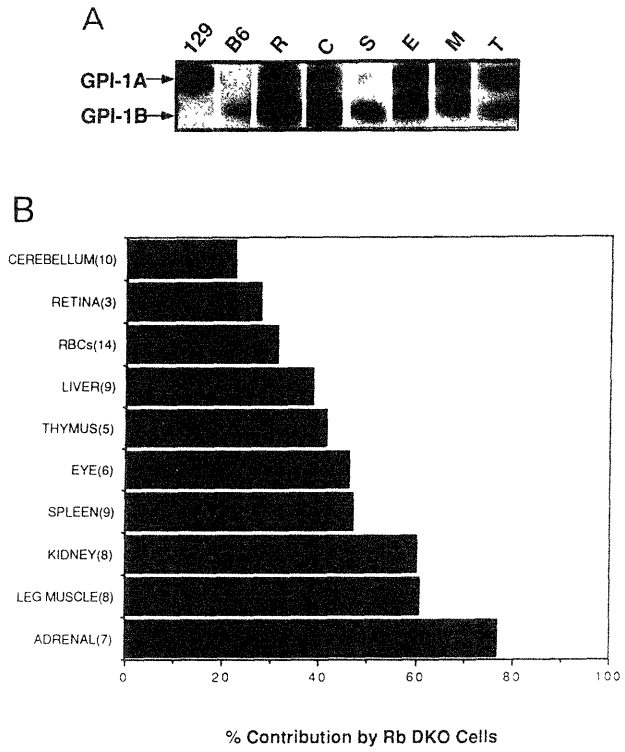

Fig. 2. GPI isoenzyme analysis (**A**) GPI activity assay. Lysates from several tissues isolated from *Rb*-DKO chimeras were separated by cellulose acetate electrophoresis and stained for GPI activity as described in Materials and methods. Tissues shown are retina (R), cerebellum (C), spinal cord (S), red blood cells (E), bone marrow (M) and tail (T). *Rb*-DKO cells (derived from strain 129/Sv) express the GPI-1A isoform, while host C57BL/6 cells produce GPI-1B. The migration of the two isoforms is shown in control lanes of 129/Sv tissue (129) and C57Bl/6 tissue (B6) and is indicated on the left. (**B**) Quantification of tissue contribution of *Rb*-DKO cells. Graph shows the average contribution (by percentage) of *Rb*-deficient cells to representative tissues isolated from *Rb*-DKO chimeras. The number of animals from which each tissue was examined is indicated in parentheses. The lowest and highest values of *Rb* −/− contribution to tissues in individual chimeras were the following: cerebellum (low of 2.5%, high of 54.4%), retina (22.9%, 30.2%), red blood cells (1.8%, 64.8%), liver (4.3%, 83.9%), thymus (22.8%, 60.3%), eye (33.2%, 61.1%), spleen (13.7%, 66.3%), kidney (49.9%, 77.9%), leg muscle (37.5%, 78.7%) and adrenal (61.2%, 97.7%). A list of the 36 different tissues tested in total is provided in Materials and methods.

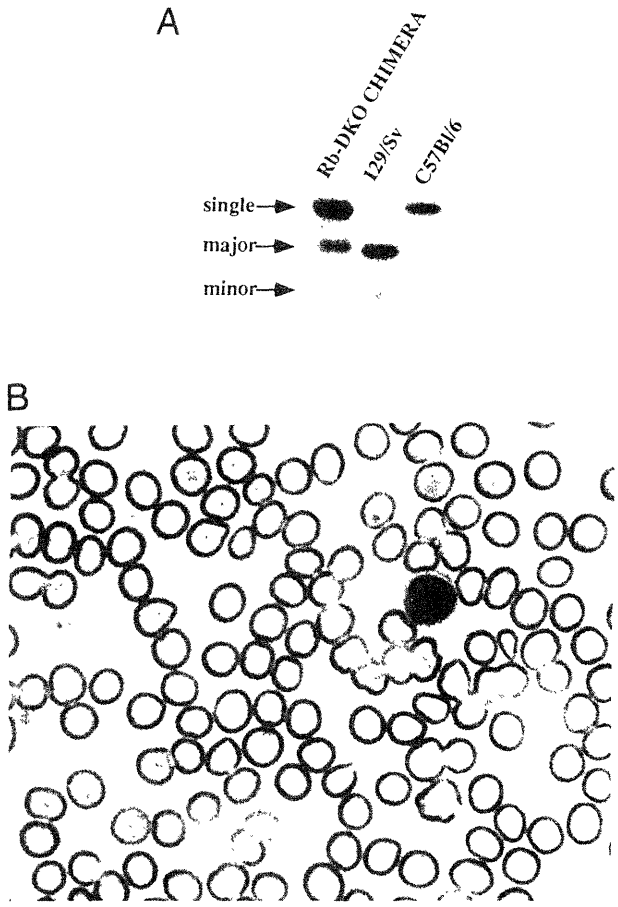

Fig. 3. Normal erythropoiesis in *Rb*-DKO chimeras (**A**) β-globin isoforms. Blood proteins from an *Rb*-DKO chimera and 129/Sv and C57Bl/6J controls were separated by cellulose acetate electrophoresis and stained as indicated in Materials and methods. The presence of *Rb*-deficient erythroid cells in the *Rb*-DKO chimeras is verified by the production of the 129/Sv-specific Hb major and Hb minor species. Hb single (produced by strain C57Bl/6 red blood cells) is also evident in the chimeras. (**B**) Normal erythrocytes. A typical blood smear (stained with Wright–Giemsa) from an *Rb*-DKO chimera shows normal red blood cells and a lack of nucleated erythrocytes. Blood smears from control chimeras generated from ES cells heterozygous for the *Rb* mutation were indistinguishable from those derived from *Rb*-DKO chimeras (data not shown).

conclude that all cell types within that tissue can differentiate in the absence of *Rb* function.

As reported previously, embryos homozygous for an *Rb* mutation die at 13.5–14.5 days *in utero* with defects in hematopoiesis and neurogenesis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Thus, it was of particular interest to examine the ability of *Rb*-deficient cells to contribute to these tissues in the *Rb*-DKO chimeras. As shown in Figure 2B, GPI isoenzyme analysis clearly demonstrates significant contribution by the *Rb*-deficient cells to at least some portions of the adult brain, spinal cord and red blood cell (RBC) compartment. The percentage of *Rb*-deficient cells in the cerebellum (and other central nervous system tissues) was consistently among the lowest of all the tissues examined (Figure 2B, data not shown). However, this pattern was also observed in chimeras made from ES cells heterozygous for the *Rb* mutation (data not shown), and, therefore, presumably reflects the relative ability of the ES cell-derived donor cells to compete with the cells of host embryo during organogenesis.

The rather extensive contribution of the *Rb*-deficient cells to the RBC compartment was unexpected in light of the observed inhibition of erythropoiesis in *Rb* homozygous mutant embryos (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). In order to confirm that the GPI-1A signal measured from the purified RBC fraction was derived from *Rb*-deficient erythrocytes (rather than some contaminating cell type), we assayed for the presence of the Hb major and Hb minor forms of β-globin produced by the Hbb^d haplotype of strain 129/sv (Whitney, 1977). As shown in Figure 3A, erythrocytes isolated from strain C57BL/6 animals produce Hb single (encoded by the Hbb^b haplotype), which is separable from the 129/sv-specific Hb major and Hb minor species. The RBC fraction isolated from *Rb*-DKO chimeras clearly contains all three β-globin forms (Figure 3A), indicating the presence of *Rb*-deficient hemoglobinized cells. Peripheral blood

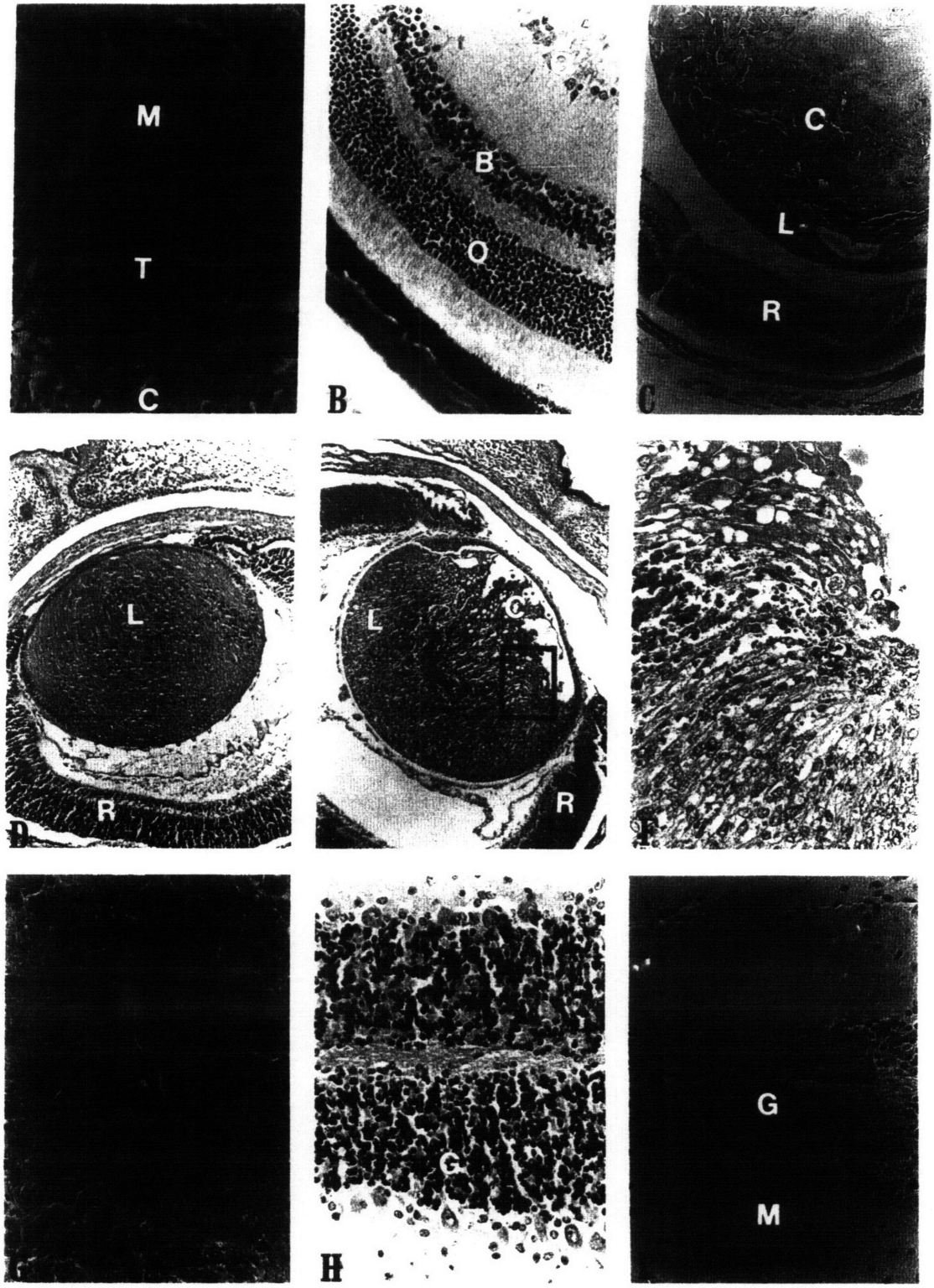

Fig. 4. Histopathology. (**A**) Hyperplastic nodule (T) in the adrenal medulla of an *Rb*-DKO chimera; normal medulla (M) and cortex (C) are indicated. (**B**) High magnification of a section through the retina of an *Rb*-DKO chimera with normal outer nuclear (O), bipolar cell (B), and ganglionic cell (G) layers. (**C**) Section of whole eye of an *Rb*-DKO chimera showing a cataract (C) in the lens (L) and normal retina (R). (**D**) Section of normal eye of a newborn control chimera with lens (L) and retina (R) indicated. (**E**) Section of eye of a newborn *Rb*-DKO chimera showing abnormal lens (L) architecture and an early cataract (C); retina (R) is also noted. (**F**) Higher magnification view of area boxed in panel E. Disorganized lens fiber cells are shown at the bottom, dying cells with pyknotic nuclei in the middle and cellular debris above. (**G**) Section of an *Rb*-DKO chimera liver showing cells with abnormally enlarged nuclei (arrows). (**H**) Normal cerebellum from a control chimera. The granular cell (G), molecular (M) and Purkinje cell layers (P) and normal Purkinje cells (arrow) are indicated. (**I**) Abnormal cerebellum in an *Rb*-DKO chimera. Ectopic and enlarged Purkinje cells are shown (arrows) along with reduced granular cell layer (G). Magnification: panels A, B and F–I, 200×; panels C–E, ~50×.

smears from the *Rb*-DKO chimeras further confirmed that the cells producing the 129/sv-specific β-globin were indeed mature erythrocytes (Figure 3B). Notably, there was no increase in the percentage of nucleated RBC in these animals compared with controls. Thus, it is clear that *Rb*-deficient erythroid cells, whose development is inhibited in an *Rb* homozygous mutant embryo, can develop normally when in the environment of wild-type cells.

Histological and pathological consequences of Rb deficiency

The analysis described above indicates a surprisingly large percentage of *Rb*-deficient cells in many tissues of the adult chimeras. However, these data do not address whether the development of these mutant cells occurred normally. In particular, it was possible that the *Rb*-deficient cells might be defective in reaching or maintaining endstage differentiation which could produce pathological and tumorigenic consequences in some tissues. Therefore, we undertook a detailed histopathological characterization of a number of tissues in the chimeric animals to determine whether their development was, in fact, fully normal. For all of the tissues examined by GPI analysis, a sample was fixed, processed and examined by standard histological methods.

For the majority of tissues analyzed, the *Rb*-DKO chimeras were indistinguishable from controls. In four tissue types, however, the presence of *Rb*-deficient cells produced obvious abnormalities. When the adrenal medullae of four *Rb*-DKO chimeras were examined histologically, all four were found to contain multiple small nodules of hyperchromatic cells, indicative of preneoplastic lesions (Figure 4A). In addition, in six of eight animals examined, areas of the cerebellar cortex had displaced Purkinje cells, most of which had enlarged nuclei (Figure 4H and I). In the affected cortical areas, the granular cell layer was also abnormally thin (Figure 4H and I). This defect in cerebellar differentiation is interesting considering the widespread neuronal cell death seen in *Rb* homozygous mutant embryos. The livers of several *Rb*-DKO chimeras also contained hepatocytes with abnormally enlarged nuclei (Figure 4G).

Finally, the most consistent abnormality observed in the *Rb*-DKO chimeras involved the lens of the eye: each of these animals developed bilateral cataracts (Figure 4C). These cataracts were characterized by posterior migration of the lens epithelium associated with bladder cell formation and, in some cases, calcification. Most animals showed evidence of cataract formation at the time the eyes opened (~1 week of age), and several newborns examined histologically already had obvious lesions in the lens. The lenses of chimeric newborns were highly disorganized and contained large numbers of pyknotic cells, indicative of apoptosis (Figure 4E and F). None of these defects were observed in control chimeras made from heterozygous ES cells. Despite a measurable contribution of *Rb*-deficient cells to the eyes, including crude retinal preparations (Figure 2A and B), we have not observed any evidence of retino-blastoma in the *Rb*-DKO chimeras. Moreover, the retinal architecture appeared normal (Figure 4B). Thus, the absence of *Rb* function alone does not lead to the outgrowth of clones of transformed cells in the mouse retina.

Pituitary tumorigenesis

At 3–4 months of age the *Rb*-DKO chimeras exhibited severe wasting and became moribund. Autopsy revealed severe adenocarcinoma of the pituitary, the same tumor observed in animals heterozygous for the Rb^{w31} mutation (Jacks *et al.*, 1992). Immunohistochemical analysis performed on three of these tumors indicated that they arose from the intermediate lobe of the pituitary. The tumors stained positively with antibodies directed against the hormones α-melanocyte stimulating hormone (MSH), β-endorphin and adrenocorticotropic hormone (ACTH) (all produced in the intermediate lobe) and not for markers specific for other lobes of the pituitary including thyroid stimulating hormone (TSH) and prolactin (Figure 5, data not shown). This pattern of staining was also observed with pituitary tumors from Rb^{w31} heterozygotes (data not shown).

The average age at which *Rb*-DKO chimeras manifested the effects of pituitary tumorigenesis was ~4 months (Figure 6). All 10 chimeras that were not sacrificed at earlier ages for other purposes eventually developed this tumor. Thus, the latency of tumorigenesis observed in the *Rb*-DKO chimeras is greatly reduced relative to the Rb^{+} heterozygotes, most of which succumb to pituitary tumors at ~9–10 months of age (Figure 6). Interestingly, in both *Rb* heterozygotes and the *Rb*-DKO chimeras, the time window during which most animals are affected is remarkably narrow and well-defined (Figure 6). Thus, it appears that the absence of *Rb* accelerates the death of the *Rb*-DKO chimeras (relative to *Rb* −/− mice) caused by pituitary tumorigenesis. Although we have also observed preneoplastic lesions in the adrenal glands of some *Rb*-DKO chimeras, it is noteworthy that there are relatively few tissues in which homozygous inactivation of *Rb* causes oncogenic transformation.

Discussion

Mutant alleles of the *RB* gene are found in a variety of human tumors, including the retinal tumors in which this gene was first characterized, osteosarcomas, carcinomas of the lung, bladder and breast (Friend *et al.*, 1986, 1987; Fung *et al.*, 1987; W.H.Lee *et al.*, 1987; Harbour *et al.*, 1988; E.Y.-H.P.Lee *et al.*, 1988; T'Ang *et al.*, 1988; Yokota *et al.*, 1988; Varley *et al.*, 1989; Bookstein *et al.*, 1990*b*; Hensel *et al.*, 1990). For this reason, the *RB* gene has been thought to function as an important negative regulator of cell proliferation in these various tissues. An even more widespread involvement in growth regulation has been suggested by the almost ubiquitous expression of this gene in the tissues of the mouse (Bernards *et al.*, 1989; Szekely *et al.*, 1992) and the growth inhibitory effects of exogenous *RB* expression (Huang *et al.*, 1988; Bookstein *et al.*, 1990a; Qin *et al.*, 1992). The RB protein is found to undergo phosphorylation in mid/late G_1 (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989) and this phosphorylation has been connected with the functioning of cyclins and associated kinases (Lees *et al.*, 1991; Lin *et al.*, 1991; Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993) Moreover, pRb has been shown to associate with a number of DNA tumor virus oncoproteins (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989) and cellular transcription factors, most notably E2F (Chellappan *et al.*, 1991; Rustgi *et al.*,

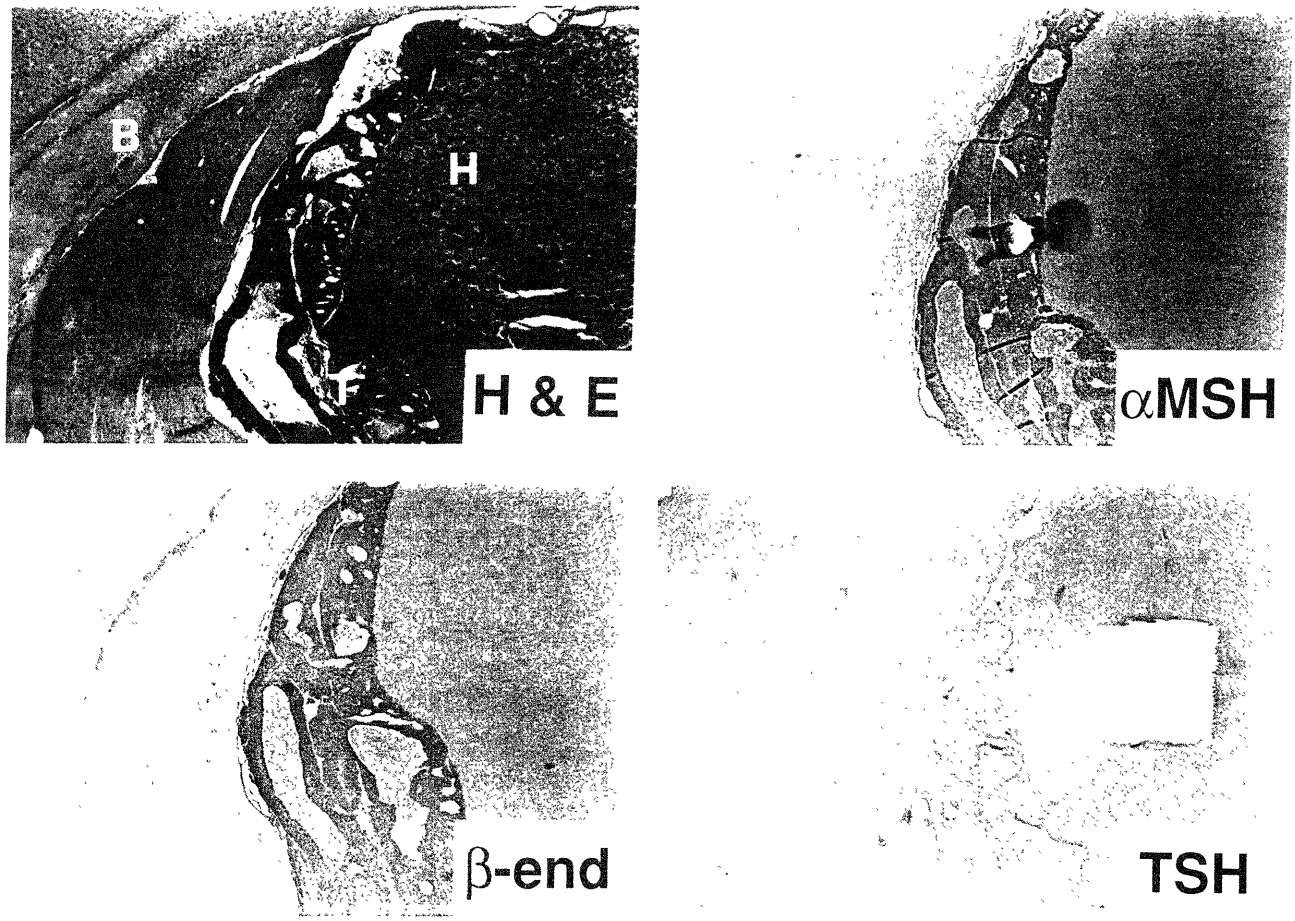

Fig. 5. Immunohistochemistry of pituitary tumors. Section of pituitary from an *Rb*-DKO chimera stained with hematoxylin and eosin (H & E) showing tumor (T), brain (B) and a large hematoma (H). Tumor tissues stain positively with antibodies against the hormones α-MSH, β-endorphin (β-end) and ACTH (not shown) produced in the intermediate lobe of the pituitary and not with antibodies for thyroid stimulating hormone (TSH) or other markers of the anterior pituitary, including prolactin (not shown).

1991; Gu *et al.*, 1993; Hagemeier *et al.*, 1993). Taken together, these various lines of evidence imply that pRb regulates progression through a critical point in the cell growth cycle.

The resulting model of pRb as an important controller of proliferation and transformation in a wide variety of cell types is difficult to reconcile with the observations presented here. The present work describes the creation of chimeric mice, many of whose tissues are morphologically normal yet contain large proportions of *Rb*-deficient cells. These mice are largely developmentally normal and survive until 3–4 months *post partum*, at which time they die from the effects of adenocarcinomas of the pituitary. In a separate series of experiments, we have also been able to produce apparently normal *Rb*-deficient B and T lymphocytes using the RAG-2 blastocyst complementation assay (Chen *et al.*, 1993). Thus, the accumulated data suggest that *Rb* is dispensable for the development and maintenance of the differentiated state of most cell types in the mouse. This extends the earlier observations which showed that mouse embryos homozygous for an *Rb* mutation develop normally until day 13 of gestation, at which point they die, apparently because of a defect in erythropoiesis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). It is important to note that while the data

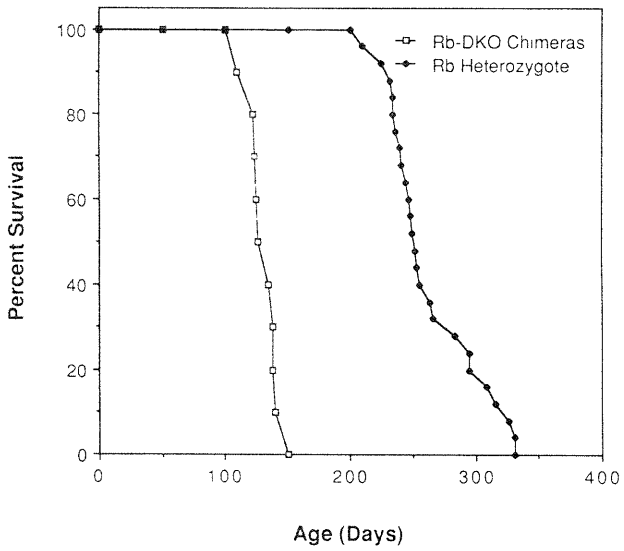

Fig. 6. Viability of *Rb*-DKO chimeras. Graph summarizes the viability of 10 *Rb*-DKO chimeras and 25 animals heterozygous for a germline mutation in *Rb*. Heterozygotes are inbred on the 129/Sv genetic background. Data points reflect the time at which animals became moribund and were sacrificed due to the effects of intermediate lobe pituitary tumors.

presented here suggest that *Rb*-deficient cells are capable of contributing to all tissues of the adult mouse, the ability of these cells to differentiate into every cell type within a given tissue cannot be determined at this time. Therefore, it remains possible that there are certain cells in the animal that cannot be formed in the absence of *Rb* function. Moreover, the survival of embryos that are composed largely of *Rb*-deficient cells may still require the contribution of wild-type cells to one or more critical cell lineages during development.

One of the more unexpected results of this analysis was the high percentage of *Rb*-deficient erythrocytes in the peripheral blood of chimeric animals. Due to incomplete differentiation *in vitro* of erythroid precursors isolated from the livers of *Rb* homozygous mutant embryos, we had previously suggested that the requirement for *Rb* in erythropoiesis was cell autonomous: that is, the function of the *Rb* gene was required within erythroid precursor cells in order for them to differentiate normally (Jacks *et al.*, 1992). However, we now consider it more likely that the absence of *Rb* function does not affect erythropoiesis by directly inhibiting differentiation of erythroid cells *per se* but, rather, by affecting the ability of other cell types to create an environment allowing survival and differentiation. For example, the absence of *Rb* function might prevent stromal cells from producing one or more critical growth, differentiation or survival factors essential for normal erythroid differentiation. In the chimeras, the wild-type cells derived from the host embryo could 'rescue' the differentiation of the mutant cells by supplying the necessary extracellular factor(s) to the microenvironment. The previously described inefficient *in vitro* differentiation of *Rb*-deficient precursors might be explained by a pre-existing defect due to their failure to receive appropriate environmental signals prior to their removal from the embryo. Furthermore, the apparently normal development of other *Rb*-deficient cell types could also be affected by the presence of wild-type cells in the chimeras.

According to Knudson's two-hit model of retinal tumorigenesis, the development of human retinoblastoma is dependent on two rate-limiting steps (Knudson, 1971), which are now understood to be the successive inactivation of two copies of the *Rb* gene (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). Since mice heterozygous for an *Rb* mutation do not develop retinoblastoma, we and others have proposed that the loss of function of *Rb* is not sufficient to transform the relevant cell type in the mouse retina (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Alternatively, the absence of retinoblastoma in these animals could reflect the relatively small number of susceptible cells in the retina of the mouse, which would be expected to decrease the likelihood of the second hit in Knudson's model. Having found that the *Rb*-DKO chimeras (in which a measurable fraction of retinal cells are already homozygous for an *Rb* mutation) also do not develop retinoblastoma, we now strongly favor the notion that the relevant cell type in the mouse is refractory to the loss of *Rb* function. This implies that the transformation of this cell type in the mouse requires additional genetic events and may explain why sporadic retinoblastoma has never been reported in mice (or indeed in any other species besides our own). Mice expressing SV40 T antigen do develop retinoblastoma at high frequency (Windle *et al.*, 1990; al-Ubaidi *et al.*, 1992), suggesting that some of the multiple activities of this viral oncoprotein beyond its ability to bind and sequester pRb (DeCaprio *et al.*, 1988) contribute to retinal cell transformation.

Mice heterozygous for the $Rb^{+/3f}$ mutation are highly predisposed to intermediate lobe pituitary tumors (Jacks *et al.*, 1992). Tumorigenesis appears to require the inactivation of the wild-type *Rb* allele, and the average age of sacrifice (from the effects of the tumor) is ~10 months. In the *Rb*-DKO chimeras, the rate of tumorigenesis is increased; these animals become moribund from the effects of the pituitary tumors by 4 months of age. Thus, as predicted from Knudson's model (Knudson, 1971), the removal of a rate-limiting step (the inactivation of the wild-type allele of *Rb*) accelerates the rate of tumor development. This increased rate could result from the more rapid formation of a single tumor focus or the combined effects of several distinct foci. By sacrificing animals at progressively earlier ages, it should be possible to determine the number of foci that contribute to tumor growth in both the heterozygotes and the *Rb*-DKO chimeras. The strikingly precipitous decline in the survival of the *Rb*-DKO chimeras during a narrow window of time indicates that few, if any, additional stochastic events are required for the transformation of the cells of the intermediate lobe of the pituitary.

Finally, given the frequent mutation of the *Rb* gene in human cancer and the proposed importance of this gene in cell cycle regulation (Weinberg, 1992), we had anticipated that seeding an animal with large numbers of *Rb*-deficient cells would result in widespread tumorigenesis but found that tumorigenesis is limited to the pituitary gland and the adrenal medulla. In order to produce more profound effects on tumorigenesis (and development) it may be necessary to inactivate simultaneously two or more members of the *Rb* family of genes which now includes the genes encoding p107 (Ewen *et al.*, 1991) and p130 (Cobrinik *et al.*, 1993; Hannon *et al.*, 1993; Li *et al.*, 1993).

Materials and methods

Creation of Rb-DKO ES cells

Heterozygous ES cells (Jacks *et al.*, 1992) were electroporated with an *Rb* targeting vector containing a hygromycin resistance gene in place of the neomycin resistance gene of the original targeting vector, described in Jacks *et al.* (1992). The hygromycin gene is under the control of the *PGK* promoter and polyadenylation sequences (te Riele *et al.*, 1990). The conditions for electroporation in the second round of gene targeting were identical to the first (Jacks *et al.*, 1992). Following electroporation, cells were plated in the absence of a feeder layer, and selected with 300 μg/ml G418, 125 μg/ml hygromycin and 2 μM ganciclovir. Colonies were selected and expanded for DNA analysis and subsequent blastocyst injection.

Southern blot analysis

Preparation of genomic DNA and Southern blotting were performed exactly as described (Jacks *et al.*, 1992) using ^{32}P-labeled probes indicated in Figure 1A.

Generation of chimeric mice

Blastocyst injection and creation of chimeric mice were performed as described (Bradley, 1987) except that eight to 10 ES cells were injected per blastocyst.

Histological analysis of tissues

Tissues were surgically removed, fixed in Bouin's solution, dehydrated in graded alcohol solutions, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin.

Immunohistochemistry

Tissues fixed in Bouin's solution were stained with polyclonal antibodies to prolactin and TSH (generously provided by Salvatore Raiti of the National Hormone and Pituitary Program at the University of Maryland School of Medicine with the National Institute of Diabetes and Digestive and Kidney Diseases), β-endorphin, α-MSH and ACTH (BioGenex Laboratories). Immunohistochemistry was performed using appropriate secondary antibodies (BioGenex Laboratories) and a biotin-streptavidin-peroxidase-based staining kit with a diaminobenzidine substrate (BioGenex Laboratories)

Retinal isolation

Crude retinal isolates were obtained by removing the cornea and lens with a circumferential incision behind the limbus. Retinal tissue was first excised along with the attached optic nerve and further dissected away from it.

Preparation of red blood cell fractions

Whole blood in anticoagulant was diluted 2-fold in phosphate buffered saline, layered onto two volumes of Ficoll–Hypaque (Histopaque-1077, Sigma) and centrifuged at 3500 *g* for 20 min at room temperature. The pellet, enriched for RBC, was collected, platelets remain at the top of the gradient while lymphocytes and monocytes are enriched at the interphase.

GPI assay

Separation and detection of GPI isoenzymes was performed essentially as described (Bradley, 1987). Titan III Zip Zone cellulose acetate plates (Helena Laboratories) were soaked in Tris–glycine buffer (25 mM Tris, 200 mM glycine, pH 8.5) for 20 min prior to application of samples. Samples were prepared by diluting tissues with ~10 vol of water, and were then crudely homogenized; cells were then lysed by three rounds of freezing and thawing. Most samples were then further diluted 10-fold with distilled water. Purified RBC were diluted a total of 1:5. Samples were electrophoresed in triplicate in a Zip Zone chamber (Helena Laboratories) for 1.5 h at 150 V at 4°C, and stained as described (Bradley, 1987). Relative levels of the two GPI isoforms were quantified by densitometry with an LKB 2222-010 UltroScan XL linear densitometer. Tissues analyzed included cerebellum, cerebrum, medulla oblongata, crude retinal isolate, epididymis, RBC, lung, tongue, bone marrow, testis, liver, uterus, optic nerve, thymus, diaphragm, colon, eye, lymphocytes and monocytes, spleen, tail, cecum, stomach, bladder, atrium, ventricle, salivary gland, small intestine, ovary, kidney, leg muscle, seminal vesicle, trachea/thyroid, prostate, esophagus, pancreas, fifth cranial nerve and adrenal gland.

Hemoglobin assay

Cellulose acetate electrophoresis of cystamine-modified hemoglobins was performed essentially as described (Whitney, 1977; Pevny *et al.*, 1991). Purified RBC were added to 75 ml of cystamine lysis buffer (12.5 mg/ml cystamine dihydrochloride, 1 mM dithiothreitol, 0.55% ammonium hydroxide) and agitated to lyse the RBC. Samples were applied to Titan III cellulose acetate plates (Helena Laboratories) (previously soaked for 10 min in an electrophoresis buffer of 0.18 M Tris, 0.10 M boric acid, 0.002 M EDTA) and run for 40 min at 300 V. The plates were placed in staining solution (1% Ponceau S, 5% trichloroacetic acid) for 10 min and rinsed in three changes of 5% acetic acid for 10 min each. Plates were then immersed in 100% ethanol for 10 min, then in clearing solution (30% acetic acid, 70% methanol) for 5 min, and finally baked at 55°C for 5 min.

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One of the most surprising observations from this work was that the presence of *Rb*-deficient cells in many adult tissues did not result in more widespread tumorigenesis. One notable exception was in the adrenal medulla, where hyperplastic lesions were observed. This suggests that most cells do not rely on the presence of the *Rb* protein for negative growth control or are capable of compensating for *Rb*-deficiency via some unknown mechanism. Perhaps the related genes *p107* and *p130* are involved in this compensation (see Appendix D).

The lack of widespread tumorigenesis also suggests that the loss of the wild-type allele of *Rb* in heterozygous mice is not a rate-limiting step during most murine tumorigenesis. This also implies that the cooperative effects of *Rb* and *p53* mutations in tumorigenesis is not due to a simple increased rate in the loss of the wild-type allele of *Rb*, since these chimeras do not develop the islet cell tumors, pinealoblastomas, and other pathology seen specifically in the *Rb*+/-;*p53*-/- mice (see Chapter 3).

Perhaps the most important observation from them these experiments the indication that at least some defects noted in *Rb*-deficient embryos could be rescued by the presence of wild-type cells. The fact that *Rb*-deficient red blood cells could be formed and were not noticeably abnormal suggested that the something in the microenvironment of the chimeric fetal liver allowed the maturation of *Rb*-deficient cells. Kay Macleod, a post-doctoral fellow in our laboratory, has extended this observation by showing that fetal livers from *Rb*-deficient embryos could reconstitute the hematopoietic systems of lethally-irradiated adult mice. This again illustrates that the defect in fetal liver hematopoiesis is at some level a non-cell autonomous defect. However, the situation is more complex than this as these reconstituted hosts die at about 4-6 months after the rescue, implying that the *Rb*-deficient cells, for whatever reason, are not fully normal.

While the GPI and hemoglobin polymorphism assays allowed the assessment of contribution of *Rb*-deficient cells to the tissues of the chimeras of the animals, no mechanism for assessing the contribution of *Rb*-deficient cells at the cellular level existed. Ideally, one would like to stain tissue

sections for the presence of *Rb*-deficient cells to conclusively prove which cell types within tissues to which *Rb*-deficient cells could contribute. Attempts at staining for *Rb*-protein were not successful (data not shown) as no good antibodies existed at that time to do good immunohistochemistry in the mouse. In fact, only one group has reported such an analysis in the mouse, and this was in the context of a very specialized system. In addition, *in situ* hybridization analysis for the presence of neomycin mRNA have proven unsuccessful to date in this and other chimeric mouse models (T. S. Shih, personal communication). Thus, to further increase the utility of this system for studying *Rb* function (as well as other genes functions) we needed to develop method to overcome this problem. Our approach to this problem is the subject of the following chapter.

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Note: Additional citations relevant to this chapter are included on page 126-127.

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CHAPTER 6

Derivation And Analysis Of Chimeras Created From *Rb*-Deficient; *ROSA -26* Positive ES Cells

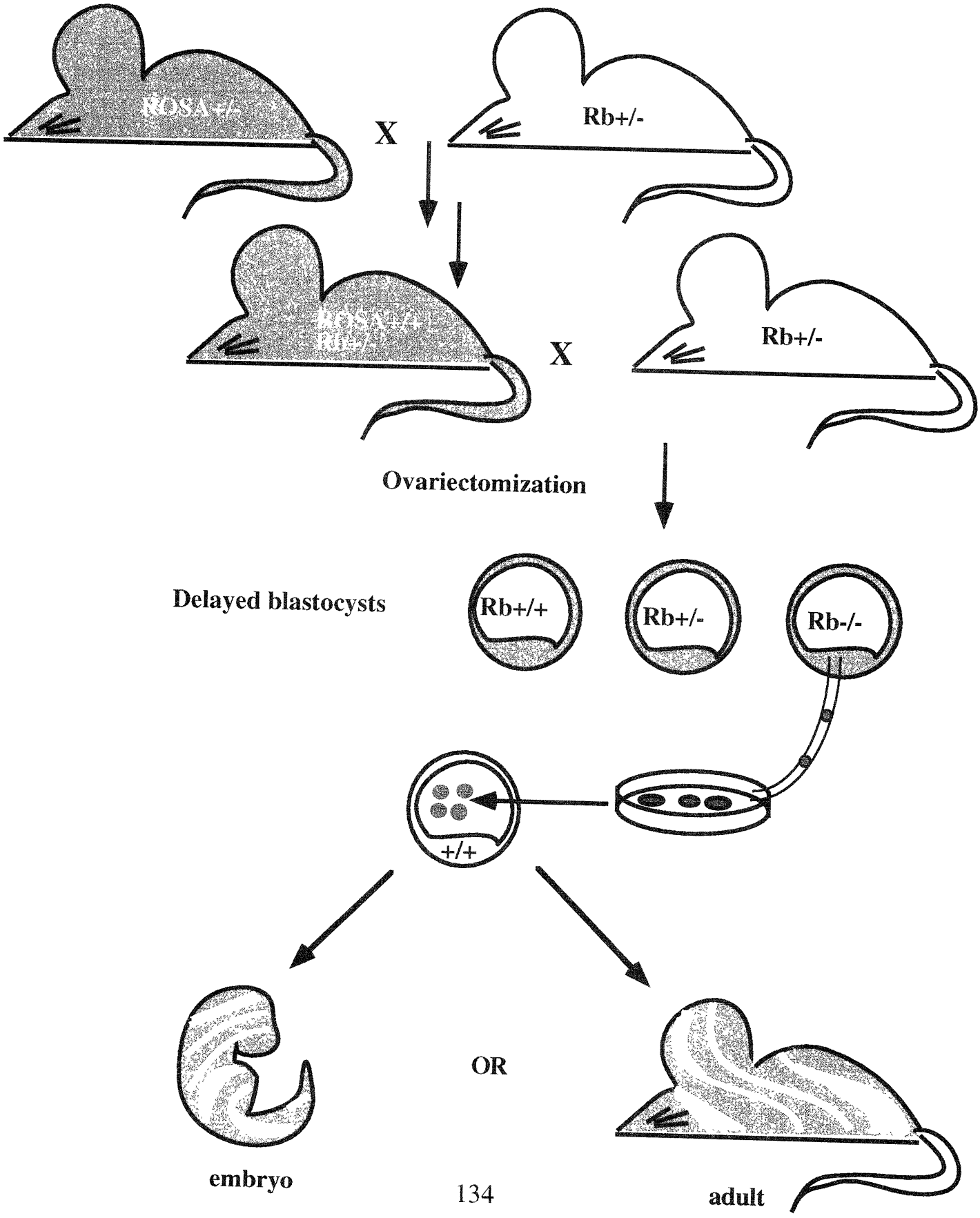
One method to identify *Rb*-deficient cells in the context of a chimeric embryos was to introduce a marker into the ES cells whose presence could be identified by some simple technique. Since the *lac Z* gene (which encodes the bacterial B–galactosidase protein) had been used in a number of contexts to assess gene expression it seemed a logical candidate for such a marker (Bonnerot and Nicolas, 1993). It has several advantages including being a stable protein, having no mammalian homolog to create potential background problems in staining, and its presence can be determined by staining with 5-Bromo-3-chloro-2-indolyl-galactosidase (X-gal), which when cleaved by B–galactosidase leaves a bright blue precipitate (Bonnerot and Nicolas, 1993).

The *ROSA-26* mouse strain was created during a experiment designed to identify genes which were expressed in developmentally interesting ways (Friedrich and Soriano, 1991). In this approach, Friedrich and Soriano designed a promoterless vector that contained a gene which encoded a fusion protein of B–galactosidase and the neomycin resistance gene product. After this vector (termed ROSA-Bgeo for reverse orientation splice acceptor BGal-neo fusion protein) was electroporated into ES cells, clones were selected in the presence of G418. These clones were then injected into blastocysts to create chimeric mice, and the chimeras subsequently bred to generate mice which contained the integrated transgene in their germline. While several of these transgenic lines were expressed in spatially and temporally specific ways, other lines (most notably *ROSA-26*) caused widespread, high levels of B–gal expression (Friedrich and Soriano, 1991). Furthermore, when this single-copy insertion was bred to homozygosity, no phenotypic affect was seen in the mice (Friedrich and Soriano, 1991).

In order to overcome the limitations of chimeric analysis outlined in the last chapter, I chose to cross *Rb* heterozygous mice to these *ROSA-26* mice described. Females from such matings were ovariectomized at 2.5 days post-coitus and injected with Depa-provera (Sigma). This prevents blastocysts from implanting in the uterus(Robertson, 1987), which normally occurs around day 4 after fertilization. Such delayed-implantation blastocysts are then recovered from the females 4 days later and plated onto irradiated mouse embryonic fibroblasts (Robertson, 1987). For reasons

not understood, such delayed blastocysts provide better production of embryonic stem cells. After 3-4 days in culture, the inner cell mass portion of the blastocyst is picked off the plate, trypsinized, and disaggregated onto fresh feeders (Robertson, 1987). The disaggregated ICMs are then monitored and colonies with the characteristic ES cell morphology are subcloned and grown up. Once these cells were isolated, they were used to create chimeric animals. This process in summarized in Figure 6-1.

Generation of ROSA^{+} Double Knockout Chimeras



Tissues and tissue sections from these animals were then stained with X-gal to identify and analyze *Rb*-deficient cells at the single cell level. It should be pointed out that while the *ROSA-26* B–galactosidase expression pattern is reported to be ubiquitous (Friedrich and Soriano, 1991), results obtained in this study suggest that it is not. In addition, subcellular localization of the B–galactosidase protein differs dramatically between cell types. Work presented in this chapter is ongoing and will be continued by others in the future.

Materials and Methods

Identification of animals carrying the *ROSA-26* transgene

Mice containing the *ROSA-26* transgene were identified in one of two ways. One method was to obtain a small piece of ear tissue with an ear punch and stain it for the presence of B–galactosidase activity. This was done by first rinsing the tissue in PBS and then placing it in staining solution (Friedrich and Soriano, 1991) at 37 degrees for 2-8 hours. *ROSA26* positive animals were identified by the ear turning blue in the stain. A second method for identifying *ROSA-26* mice is based on a PCR assay. Tail DNA isolated as described above was assayed in a PCR reaction using the following primers (ROSA-F=5'-TTTCCACAGCTCGCGGTTG-3' and ROSA-R=5'-GATCCGCCATGTCACAGA-3'). The conditions of the PCR reaction (except for the primers) were identical to those described in Chapter 3 and Appendix A for *p53* PCR genotyping. It should be noted that both methods only allow identification of animals carrying the transgene and do not allow identification of whether the carriers are heterozygous or homozygous carriers of the single copy transgene.

Derivation of ES cell clones from intercrosses

Animals carrying the desired genotypes (in this case *Rb* heterozygotes carrying the *ROSA-26* transgene were crossed and pregnancy detected by the presence of a vaginal plug. The date of

vaginal plug detection is noted as day 0.5. At day 2.5 the female is ovariectomized and injected with 0.1 ml of 10 mg/ml Depa-Provera; both of which inhibit the implantation of blastocyst into the uterus (creating delayed implantation blastocysts) (Robertson, 1987). At day 6.5, the females are sacrificed and the uterine horns flushed with blastocyst media (Robertson, 1987). The individual delayed blastocysts are then plated onto individual 24 well wells (coated with 0.1% gelatin) with ES feeders previously plated on them at a density of 1 x 10^6 cells per well. These ES feeders were created by deriving murine embryonic fibroblasts (carrying a neomycin resistance allele) and irradiating them with 6 grays of ionizing radiation. After 3-4 days in culture (in ES media) the delayed blastocysts have attached to the feeders and the inner cell mass (ICM) has grown out from the site of attachment. ES media is then removed from the well and replaced with PBS, after which the ICM is removed from the well with a finely pulled glass pipette or plastic pipette tip and added to approximately 50 microliters of ES trypsin (Robertson, 1987). After being placed at 37 degrees for 5-10 minutes the ICM is further disaggregated into single cell suspension by repeatedly being pulled up and down into a pipette. The suspension is then added to a fresh gelatinized 24 well well containing ES feeders as before. The wells are then monitored over the course of the next 10 days and colonies with the appropriate ES cell morphology are removed from the well in a manner analogous to how the ICM was disaggregated (described above) and plated onto fresh wells with ES feeders and expanded. Upon expansion, cells were frozen in liquid nitrogen in ES freezing media (Robertson, 1987). Expanded clones were also cultured in the absence of ES feeders on gelatinized plates in order to obtain DNA samples for genotype analysis as described above.

Identification of clones

The status of *Rb* genotype was initially determined via a PCR assay previously described and confirmed via Southern blot analysis. *ROSA-26* status was determined by a PCR assay using the following primers and conditions. Determination of the sex of the cell lines was determined via a PCR based assay with primers directed against the Y specific gene *Zfy* (Page et al., 1987). The

presence of a *Zfy* specific band in the assay was interpreted as the cell line being male while the absence of the band was interpreted as the cell line being female.

Preparation of ES cells for blastocyst injection

ES cell clones were thawed onto gelatinized plates in the absence of ES feeders. (Wells or plates were gelatinized by pipetting 0.1% gelatin on them for five minutes and then aspirating off the gelatin.) After thawing the cells were grown for at least 18 hours in ES media supplemented with LIF (Robertson, 1987). When blastocysts were ready for injection (see below), the ES cells were trypsinized, pelleted, and resuspended in blastocyst media (Robertson, 1987) at a concentration of 60,000 to 100,000 cells per ml.

Blastocyst isolation

Blastocysts were isolated from females treated in one of two ways. Naturally mated C57/Bl6J females (over 7 weeks of age) were mated to males of the same strain. Three days after the detection of the vaginal plug, the females were sacrificed and blastocysts isolated by removing the uterus and flushing it with blastocyst media. Blastocysts were also isolated from superovulated females (Fraser, 1993). For superovulation, 3-6 week old C57/Bl6J females were injected intraperitoneally with 5 IU of pregnant mare serum. Forty-eight hours later the females were intraperitoneally injected with 5 IU of human chorionic gonadotrophin and housed with males of the same strain. Females were then treated in the same manner as described above after the detection of the vaginal plug the next morning.

Injection

Blastocyst injection was performed essentially as previously described (Bradley, 1987). For these experiments (unless otherwise noted), 8-12 ES cells were injected into each blastocyst.

Implantation

Implantation was done as previously described (Bradley, 1987). For these experiments between 6-9 blastocysts were implanted into each uterine horn of pseudopregnant Swiss Webster female mice (Taconic Farms).

Whole mount staining and vibratome sectioning of chimeric tissues

Another method used early in the analysis of these chimeras was to utilize a vibratome to cut fresh pieces of tissue into thin slices which could then be stained for B–galactosidase activity. In this method, embryos or tissues were immersed in fix solution (see below) for 30-45 minutes and then washed three times in wash solution (see below) for 10 minutes per wash. The tissues were then placed in stain solution (see below) either at room temperature for 15-18 hours or at 4 degrees Celsius for 24-48 hours. If desired, tissues were then processed for paraffin embedding and sections cut at 5 microns on a microtome. Sections were then deparaffinized, counterstained with nuclear fast red or eosin, dehydrated and mounted with coverslips (see Chapter 4). While this method was useful for some applications, the inability of the X-gal staining solution to penetrate more than a few microns into the tissue necessitated the use of frozen section staining in the majority of cases.

Solutions for Lac Z staining (M. Henkemeyer and T. Pawson, personal communication)

Phosphate buffer (pH=7.3)

115 ml 0.1M sodium phosphate monobasic
385 ml 0.1M sodium phosphate dibasic

Fix solution

0.4 ml 25% Gluteraldehyde
2.5 ml 100 mM EGTA (pH=7.3)
0.1 ml 1 M magnesium chloride
47.0 ml Phosphate buffer (pH=7.3)

Wash buffer

0.4 ml 1 M magnesium chloride
2.0 ml 1% sodium desoxycholate
2.0 ml 2% NP-40
195.6 ml 0.1 M Phosphate buffer (pH=7.3)

Stain solution

2.0 ml 25 mg/ml X-gal dissolved in di-methyl formamide
0.106 g potassium ferrocyanide
0.082 g potassium ferricyanide
48.0 ml Wash buffer

Generation of frozen sections

Pregnant females at various stages of gestation were sacrificed and the embryos removed via cesarean section and placed into phosphate buffered saline (PBS). A small piece of tissue was removed from the tail of the embryo and stained in X-gal stain to identify chimeric embryos prior to cutting frozen sections. The remainder of the embryo was placed in 4% paraformaldehyde at 4 degrees Celsius for 30 minutes. The embryos were then washed in PBS (again at 4 degrees Celsius) for 30-45 minutes during which time the PBS was changed three times. After this, the embryos were placed in PBS containing 30% (weight/volume) sucrose at 4 degrees Celsius for at least 18 hours. The embryos were then prepared for cryostat sectioning by immersing them in OCT (Tissue-Tek; Elkhart, IN) and then freezing them in a beaker of isopentane immersed in liquid nitrogen. The embryos in OCT were then attached to a cryostat chuck and frozen sections were cut at a thickness of 5-10 microns. Prior to X-gal staining the sections were post-fixed in 4% paraformaldehyde in PBS for five minutes and then washed twice in PBS (5 minutes each time). Sections were then incubated in X-gal stain solution overnight at room temperature in a dark, humidified container. X-gal stain was the following: (PBS with 1 mg/ml 4-chloro-5-bromo-3-indolyl-B–galactoside (X-gal), 4 mM Potassium ferrocyanide, 4 mM Potassium ferricyanide, and 2 mM magnesium chloride). After the staining was completed the sections were washed in PBS

and then rinsed in distilled water. The sections were then counterstained with nuclear fast red for 5 minutes and then rinsed in distilled water, followed by rinses in increasing grades (80, 90, and 100%) of ethyl alcohol. The slides were then rinsed twice in xylene and coverslips mounted with Permount (Fisher Scientific).

BrdU staining

Staining for BrdU incorporation was performed as previously described (see Chapter 3). Pregnant females were injected with 10 microliters per gram body weight of BrdU/FdU solution (3 mg/ml BrdU; 0.3 mg/ml FdU in PBS) one hour prior to sacrifice. Embryos were then recovered and processed either for frozen or paraffin sections. Paraffin embedded sections were analyzed for the incorporation of BrdU as described while frozen sections were treated similarly except that deparaffinization and rehydration steps were not required.

Results

Derivation of *Rb*-deficient *ROSA-26* ES cells

17 females were ovariectomized and 56 blastocysts recovered from them. From these 56 blastocysts, 16 independent ES cell clones were established. Of these 16, 3 clones were determined to be *Rb-/-* and contain the *ROSA-26* transgene. All three were also male. Two of these lines (ES2 and ES4) were used to create chimeric animals. Both lines allowed creation of good chimeras and analysis of these chimeras showed that they were phenotypically indistinguishable. In addition to these two lines, a *Rb+/-;ROSA-26* positive male ES cell line (ES6) was also successfully used to create chimeras. Test breeding of these chimeras showed that they were capable of passing the ES cell genome through the germline with high frequency. Also, a line of wild-type male ES cells carrying the *ROSA-26* transgene was used to create chimeric

animals. This cell line (BS1) was derived and characterized with Shane Shih in the process of similar experiments he was performing to study *Nf-1* gene function (Jacks et al., 1994).

Analysis of Chimeric Embryos

Examination of X-gal stained frozen sections from embryos (both control and wild-type chimeras) confirmed that *Rb*-deficient cells could contribute to most cell lineages. One limitation to the analysis is that the *ROSA-26* transgene does not appear to be expressed in enucleated red blood cells when examined with these methods, despite the fact that previous FACS analysis had suggested that it was expressed ubiquitously through all stages of hematopoietic development.

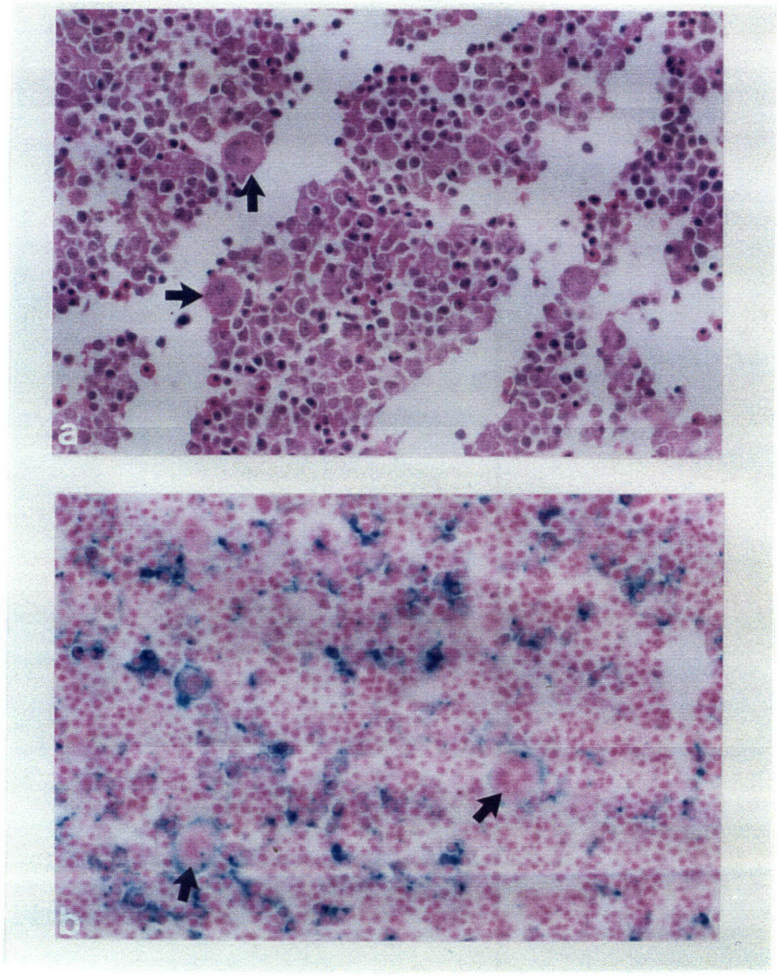
Examination of livers from days 12.5 to 14.5 showed that *Rb*-deficient (X-gal staining) fetal hepatocytes, stromal fibroblasts, and erythroid progenitors were present and appeared normal. One obvious difference between *Rb*-DKO and wild-type ES cell derived chimeras was that the fact that *Rb*-DKO chimeras exhibited highly elevated levels of megakaryocytes in their fetal livers (Figure 6-2). The vast majority of these megakaryocytes stained positive for X-gal indicating that *Rb*-deficiency in the liver may cause a cell autonomous defect at least at one level. Livers from these chimeras have also been utilized in a collaboration with Kay Macleod to successfully rescue the hematopoietic systems of lethally-irradiated adult animals. Analysis of these rescued animals is ongoing.

Figure 6-2

Analysis of 13.5 day fetal liver

(Top) Hematoxylin and eosin stained section of a fetal liver from a *Rb*-DKO chimera showing the presence of megakaryocytes (arrows).

(Bottom) X-gal stained section of another *Rb*-DKO chimera fetal liver illustrating that megakaryocytes are X-gal positive.

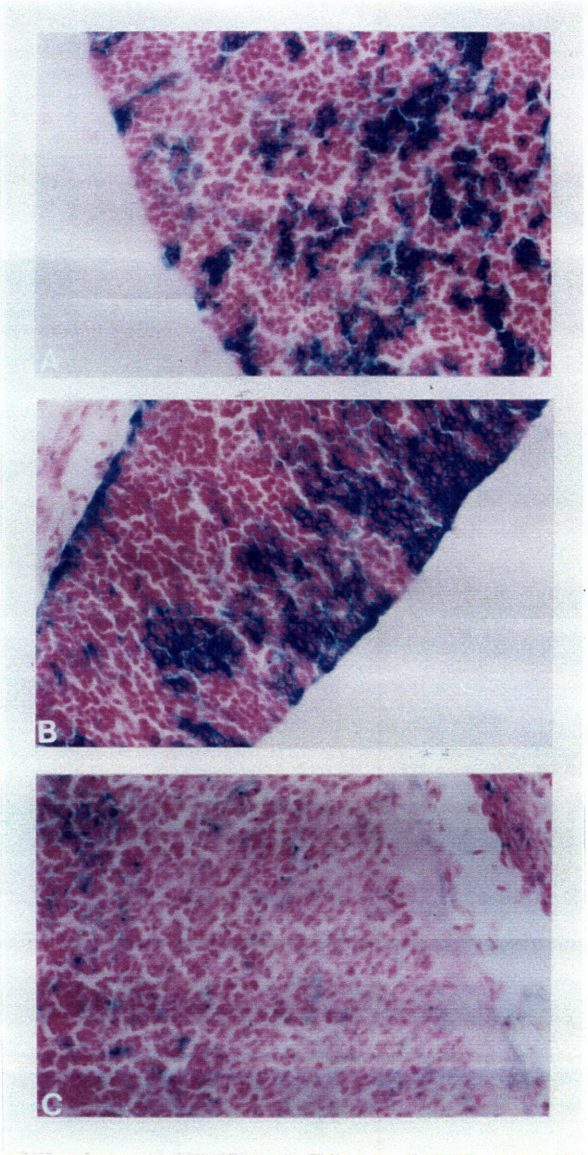


One other obvious *Rb*-associated defect to analyze in the context of these chimeras was neurogenesis. As discussed previously, *Rb*-deficient embryos have a high degree of ectopic proliferation (measured by BrdU incorporation) associated with cell death in the central and peripheral nervous system. X-gal staining analysis of *Rb*-deficient chimeric embryos clearly shows the presence of normal appearing *Rb*-deficient cells in all parts of the central and peripheral nervous system (Figure 6-3 and data not shown). Cell staining in the forebrain and midbrain showed X-gal staining throughout the cytoplasm of the cell, while staining in the hindbrain, spinal cord, dorsal root ganglion, and trigeminal ganglion was localized to specific sites in the cytoplasm. This localization difference was also seen in chimeras derived from wild-type ES cells.

Figure 6-3

Analysis of *Rb*-DKO fetal chimera central nervous system

X-gal stained sections of forebrain (top), midbrain (middle), and hindbrain (bottom) clearly showing the presence of *Rb*-deficient cells.



Several litters of chimeric animals were exposed to BrdU for one hour prior to their isolation. In order to assess whether *Rb*-deficient neurons are still proliferating abnormally in the context of a chimeras a collaborative effort between Kay Macleod and I to more fully characterize the differences between *Rb*-deficient neurons in chimeras and germline Rb-deficient animals is ongoing. This analysis will focus on the analysis of cell proliferation, apoptosis, and examination of expression of potential *Rb* targets. Preliminary analysis has revealed that neurons in the chimeras exhibit a cell autonomous defect in that *in situ* hybridization analysis reveals highly elevated levels of *Cyclin E* mRNA expression. Expression analysis of other relevant genes is ongoing.

Analysis of Adult Chimeric Tissues

Staining patterns of representative adult tissues are discussed below.

Adrenal

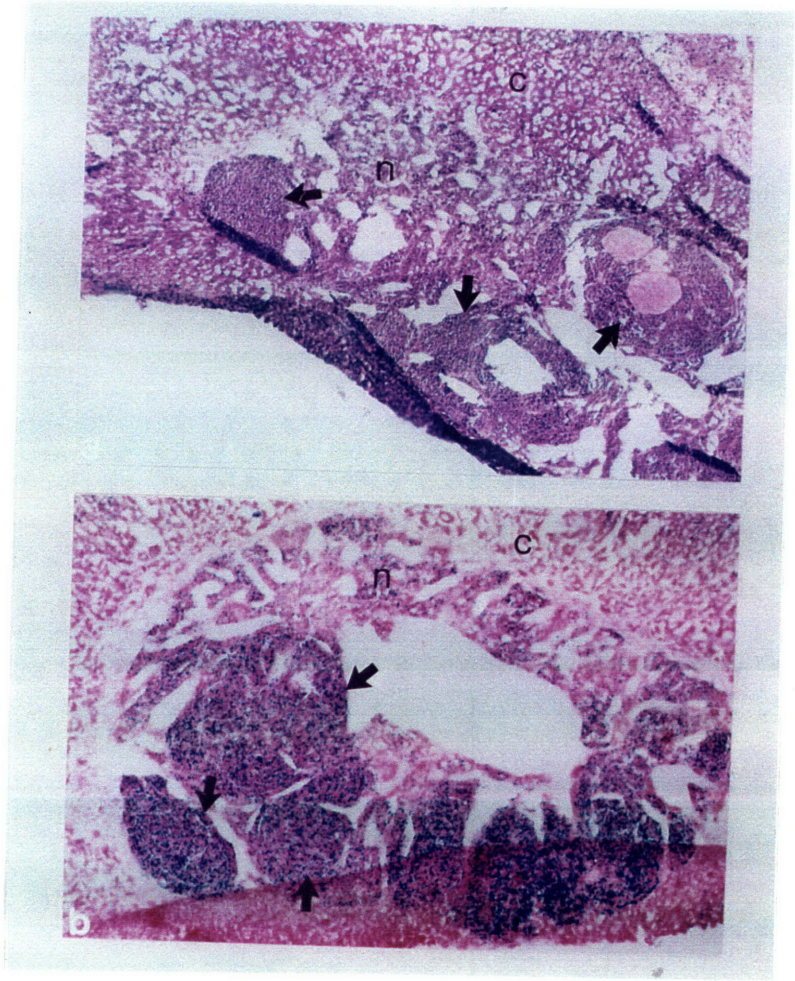
As noted by the relatively high contribution of *Rb*-deficient cells (as measured by the GPI assay), *Rb*-deficient cells contributed significantly to both the cortex and the medulla. Staining in the cortex was of a speckled nature, while staining in the medulla was more punctate. Significantly, the pheochromocytomas present in *Rb*-DKO chimeras appear to be exclusively comprised of *Rb*-deficient cells. Areas of medulla which appear normal have contribution of both X-gal positive and negative cells. These date indicate that *Rb*-deficiency is necessary, but not sufficient, for the induction of pheochromocytomas.

Figure 6-4

Adrenal X-gal staining in adult *Rb*-DKO chimeras

(Top) Hematoxylin and eosin stained section of adrenal gland showing normal cortex (C), normal medullary tissue (n), and adrenal medullary hyperplasia (arrows).

(Bottom) X-gal staining of the same adrenal gland shown above (note that these are not adjacent sections). Areas of adrenal medullary hyperplasia are composed of all X-gal positive (and thus *Rb*-deficient cells) while normal medullary tissue is a mixture of X-gal positive and negative cells.



Cerebellum

Analysis of *Rb*-DKO chimeras showed that X-gal positive staining was mainly restricted to cells of the Purkinje cell lineage. No X-gal positive cells could be found in the granular cell layer. X-gal positive Purkinje cells always appeared to be enlarged and some were ectopically placed, while X-gal negative cells always appeared to be normal in terms of placement and size. These data illustrate that the Purkinje cell defect in *Rb*-DKO chimeras is probably due to a cell autonomous defect caused by *Rb*-deficiency (Figure 6-5E and F).

Choroid plexus epithelium

Rb-DKO chimeras exhibited high levels of contribution to choroid plexus epithelium. X-gal positive cells displayed strong staining uniformly through the cell (Figure 6-5A and B).

Liver

Staining of hepatocytes showed contribution to this lineage. Staining was uniform throughout the cytoplasm, but not present in the nucleus (Figure 6-6D).

Lung

Strong contribution of *Rb*-DKO cells was noted in the bronchial epithelium with staining uniform throughout the cytoplasm of the cell. X-gal positive cells were also noted in the alveolar sacs, although staining was less intense relative to the bronchial epithelium (Figure 6-6A). Interestingly, lung tissues stained via the whole mount or vibratome method stained positive with X-gal but those processed for frozen sections did not.

Retina

Very low contribution of *Rb*-deficient cells was noted in the retina. Rare X-gal positive ganglion cells were present (Figure 6-6C). Analysis of retinas isolated from chimeras derived from wild-type ES cells is ongoing.

Lens

Because of the disorganized lens structure associated with cataracts, it was difficult to identify individual cells staining positive for X-gal. However, obvious positive activity was observed in cellular debris in the lens (Figure 6-5C).

Cartilage

Both cartilage in the tracheae and in other parts of the body showed good levels of *Rb*-deficient (X-gal positive) cells (Figure 6-6E).

Skeletal muscle

Strong positive staining of skeletal muscle was noted with staining present throughout the cytoplasm of individual muscle fibers (Figure 6-6B). Again, like the lung tissue, staining worked for the whole mount/vibratome method but not with frozen sections.

Cerebrum

Staining of individual cells in the cerebrum was noted (Figure 6-5D).

Kidney

Staining of kidney tubules was evident (data not shown).

Prostate

Strong staining of prostate epithelium was noted when the whole mount method of staining was used (data not shown).

Figure 6-5

Representative X-gal staining of tissues from chimeras derived from ES2 and ES4 injected blastocysts

A and B. Choroid plexus epithelium (100X). Note the strong staining throughout the cytoplasm.

C. Chimeric lens. A obvious cataract (C) is present. Positive staining is seen in the cellular debris within the lens capsule.

D. Cerebrum. Staining of cells within the cerebrum of a 4 month old chimeric animal.

E and F. Staining of cerebellum of chimera. Low power view in E (40X) and high power view in F (100X). Granular cell layer (g), molecular layer (m), and Purkinje cell layer (p) are noted. Note in F that several large Purkinje cells including an ectopically placed one (arrow) are *Rb*-deficient. The lack of staining in the granular layer can not be ascribed to a phenotype associated with *Rb*-deficiency as no staining is seen in granular cells from control mice.

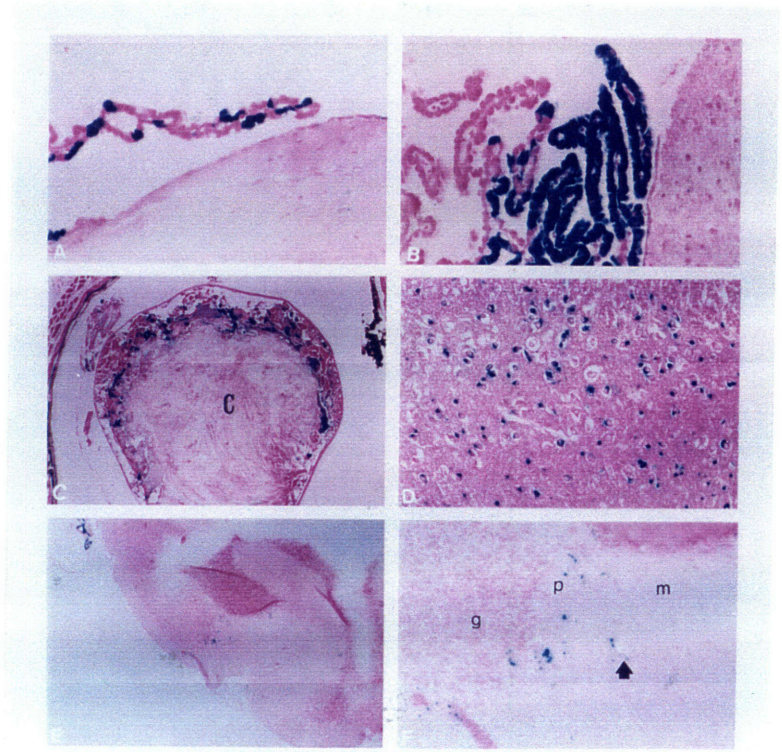

A
B
C
C
D
p
m
g

Figure 6-6

Representative X-gal staining of tissues from chimeras derived from ES2 and ES4 injected blastocysts

A. High power view (100X) of lung. Note the obvious presence of large numbers of positively staining cells in the bronchial epithelium (surrounding the lumen of the large bronchiole). An arrow notes an *Rb*-deficient cell in the alveolar spaces.

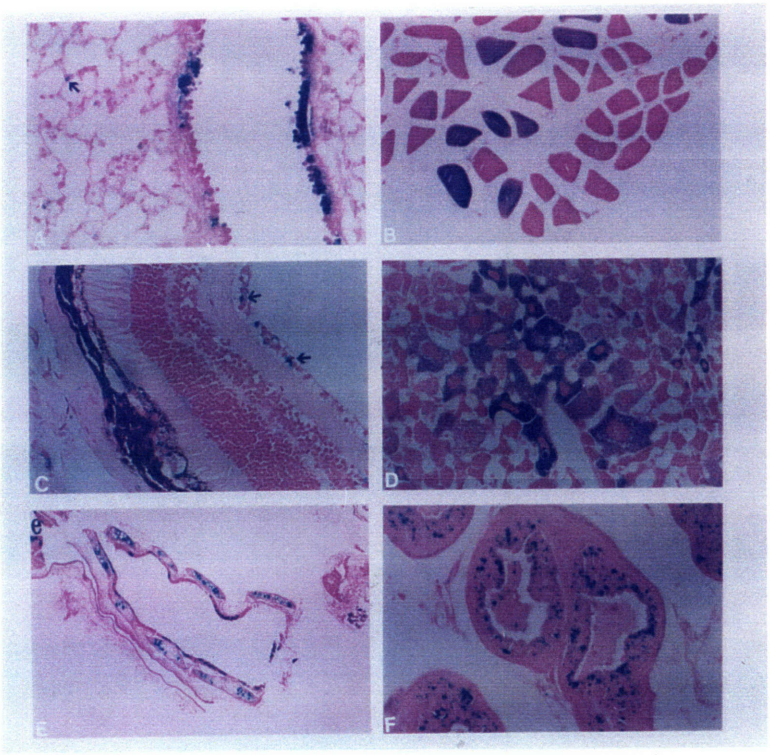
B. Skeletal muscle (100X)

C. Retina (100X). Positively staining cells in the ganglion layer are present.

D. Liver (100X)

E. Tracheae (40X)

F. Testis (100X)



Discussion

The development of the ROSA chimera system for the analysis of chimeric tissues has allowed us to begin to address the issues raised by the initial chimeric analysis (see Chapter 5) in more detail. The ability to mark individual cells within the fetal liver and the nervous system of the chimeras should go a long way towards understanding the cell autonomous defects seen in both tissues in *Rb*-deficient embryos.

Cell autonomous phenotypes in adult chimeras

The use of these ES cells has allowed us to address whether some of the effects previously noted in the chimeric adults were due to cell autonomous effects of *Rb*-deficiency. In the case of the Purkinje cell defect, the analysis supports the conclusion that *Rb*-deficiency is associated with the Purkinje cell defect as all abnormal Purkinje cells noted did stain positive for X-gal. In addition, the analysis of adrenal medullary hyperplasia shows that this pathology is associated with *Rb*-deficiency.

Non-cell autonomous phenotypes

The work presented here also reinforces the concept that both the hematopoietic and neurological defects associated with *Rb*-deficiency can be rescued in the context of wild-type cells in the developing chimera. Interestingly, in the case of the developing nervous system discussed above, *Rb*-deficient neurons in chimeras still have altered states of gene expression but are present in significant numbers without the highly elevated levels of neuronal cell death seen in embryos homozygous for a germline mutation in the gene. Understanding the basis for this difference

between *Rb*-deficient cells in the constitutionally homozygous mutant embryos and the chimeras will be one of the greatest challenges to emerge from this work.

Other uses of chimeras

The characterization of chimeras derived from these cells has allowed us to extend our previous observations (see Chapter 5). Perhaps more importantly, it has allowed us to further develop the chimera system as one which can used to test models of *Rb* function as well as derived and identify unique reagents to perform *in vitro* experiments.

One of the best examples of such work is a collaboration with Hong Yang, a graduate student in the laboratory of David Livingston at the Dana Farber Cancer Institute. Based on earlier reported work that expression of a wild-type human *RB* gene can rescue the embryonic lethality seen in *Rb*-deficient mice, Hong has taken the *Rb*-deficient cell lines described above (ES2 and ES4) and electroporated different versions of a human *RB* cDNA minigene (expressed behind a human *RB* promoter) into them. Clones which demonstrate expression of the introduced *RB* gene are then used to create chimeric animals. These chimeras are then analyzed for the defects seen in *Rb*-DKO chimeras (cataracts, pituitary tumors, adrenal medullary hyperplasia, etc.). This system allows an examination of the functions of different domains in pRB in a physiologically relevant setting.

In addition analysis of *Rb*-deficient cells in the context of the chimeras, these animals have provided valuable reagents for study of *Rb* function in tissue culture. One example of this is work being performed by Mark Day (Univ. of Michigan) and Gerald Cunha (UCSF). Initial analysis showed that *Rb*-deficient cells were capable of contributing to the prostate epithelium of adult chimeras (data not shown). Animals I provided were used as a source for *Rb*-deficient prostate epithelial cells which can be grown *in vitro* and used to examine the role of *Rb* in the control of prostate growth and differentiation. Along these same lines, I have provided mice to Oliver

Bogler, a post-doctoral fellow in Webster Cavenee's lab from which *Rb*-deficient astrocytes have been isolated and will be characterized.

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CHAPTER 7

Generation of ES Cells Deficient for Both *Rb* and *p53*

In order to further evaluate the role of *Rb* and *p53* in tumorigenesis, ES cells deficient in both *Rb* and *p53* were derived. The generation of chimeras from these ES cells is ongoing and will continue after I graduate.

The generation of these ES cells was considerably more challenging than the derivation of other ES cell lines due to several factors. The most straightforward manner to isolate these ES cells is to generate them from *Rb+/-;p53-/-* crosses. The most limiting factor was that *Rb+/-;p53-/-* animals are difficult to generate and maintain. As discussed in Chapter 3, *Rb+/-;p53-/-* animals die in the first six months of life from the affects of several tumors. Those animals which do breed are often adversely affected by the presence of these tumors, diminishing their reproductive performance. In addition, females which do breed and are used for the ES cell generation procedure often are not in good enough physical shape to survive the necessary surgical procedures.

Despite the difficulties outlined, I was able to generate one ES cell line which was deficient for both *Rb* and *p53*. Unfortunately, this cell line did not carry the *ROSA-26* transgene and furthermore did not carry the agouti allele, which necessitates Southern blot analysis to identify chimeric progeny. Chimera generation from this cell line has been initiated.

In an attempt to bypass the difficulties associated with the generation of ES cell lines in the manner described in Chapter 4, I also tried to generate *Rb-/-;p53-/- ROSA-26* containing ES cells through manipulation of cells in culture. Cell lines ES2 and ES4 (both *Rb*-deficient and carrying the *ROSA-26* transgene) were electroporated with the targeting vector *p53* Hygro KO. This vector is similar to the one described in Appendix A which used to create *p53*-deficient mice except that it contains a hygromycin resistance gene in place of a neomycin resistance gene. This was necessary because the ES2 cells were already resistant to G418 and another selectable marker was needed to identify successfully electroporated clones. These electroporated cells were then selected in media containing hygromycin and gancyclovir and subsequently analyzed to identify clones with a

homologously targeted *p53* allele. These successfully targeted clones were then subjected to selection in high concentrations of hygromycin to attempt to induce gene conversion of the remaining *p53* wild-type allele to the mutated allele. This would presumably double the concentration of the hygromycin resistance gene in the cells and allow for survival in higher hygromycin concentrations. Such protocols have been used to induce gene conversion with neomycin resistance genes, but have not been reported for hygromycin. Preliminary experiments done on the *p53* heterozygously targeted cells described above have failed to yield clones with the desired gene conversion event. Nevertheless, these cells should be valuable in generating *Rb-/-;p53-/- ROSA-26* containing ES cells either by further examining the possibility of inducing gene conversion with increased concentrations of hygromycin or by retargeting the remaining wild-type allele of *p53* with a puromycin resistance gene based vector.

Materials and Methods

Most methods used in the isolation of ES cells deficient for both *Rb* and *p53* are identical to those described above. Those not previously described follow.

Electroporation of *Rb-/-* ES cells with *p53* hygro targeting vector

ES cells were electroporated as described previously. The *p53* targeting vector was prepared similarly to that described in Appendix A except that the hygromycin resistance gene was substituted for the neomycin resistance gene. 36 hours after the electroporation, cells were exposed to hygromycin (0.125 ug/ml) and gancyclovir (2 micromolar) for 7 days. Forty-eight hours after selection was stopped, individual colonies were picked and trypsinized and expanded on ES cell feeders. When colonies had grown to sufficient numbers, a portion were frozen while the remainder were used to isolate DNA to screen for homologous recombinants. Clones were screened for homologous recombinants in the same manner as described in Appendix A.

Selection in presence of higher hygromycin concentrations

The two clones identified as having a homologous recombination event in the *p53* allele were plated on 10 cm gelatinized plates (1.0×10^4 cells were plated on each plate). After 36 hours individual plates were exposed to ES media with the following concentrations of hygromycin (in ug/ml): 0, 0.5, 1.0, 1.5, and 2.0. Selection conditions were maintained for 7 days after which individual clones were isolated and Southern blot analysis done as described above. In some cases, *p53* PCR analysis was performed to assess the genotype of ES cell clones.

Results

Of 17 females which survived the ovariectomization procedure, 45 blastocysts were recovered and 5 ES cell lines derived from them. The one *Rb-/-;p53-/-* ES cell derived (2-17 #1) was used to generate chimeric animals as described above. The analysis of offspring derived from blastocyst injections with these ES cells is more complicated than others, due to the fact that the ES cells are homozygous for the C57Bl/6 agouti locus and do not carry the *ROSA-26* transgene. In the first round of analysis of the potential of these ES cells to generate chimeric animals, 20 blastocysts were injected and implanted (along with 10 uninjected carrier blastocysts). 10 animals were born as a result and were examined for chimerism by Southern blot for the presence of the *Rb*-mutant allele. None of the 10 were found to be chimeric. Further rounds of injections are ongoing.

Initial retargeting of ES2 ES cells

One hundred seventy clones were isolated from the electroporation of ES2 cells with the *p53* hygro KO vector. Southern blot screening revealed that two of the first 16 clones analyzed contained the desired homologous recombination event. These clones were labeled 2-19 #17 and 2-19 #18.

Selection of clones in increased hygromycin concentrations

The number of colonies remaining on each plate after selection is shown below. Ten thousand cells were initially plated on each plate.

Table 7-1. Selection of ES cells in increased hygromycin concentrations

Clone 2-19 #17

Hygromycin concentration	# Colonies
0 mg/ml	422
0	450
0.5	398
1.0	216
1.5	149
2.0	25

Clone 2-19 #18

Hygromycin concentration	# Colonies
0 mg/ml	484
0	508
0.5	452
1.0	396
1.5	230
2.0	24

Southern blot and PCR analysis revealed that none of the colonies examined (including all 48 which survived the 2.0 ug/ml selection had undergone the desired gene conversion.

Discussion

In order to better understand the cooperative effects between *Rb* and *p53* mutations in murine carcinogenesis, I have derived ES cells deficient in both genes. These cells are now being used to generate chimeric animals for analysis. Several potential experiments can be performed with these cells to enhance our understanding of the functions of both genes. One obvious line of inquiry is to examine adult chimeric animals for tumorigenesis in the tissues where *Rb+/-;p53-/-* exhibit tumors or pathological abnormalities (pineal gland, islet cells of the pancreas, bronchial epithelium, retina).

I have also made progress towards the generation of a cell line which is deficient for both *Rb* and *p53* and carries the *ROSA-26* transgene (Friedrich and Soriano, 1991). As described in the results section of this chapter, *Rb*-deficient *ROSA-26* containing cells have been generated which are heterozygous for a *p53* targeted mutation. Initial attempts to generate *p53*-deficient cells from these clones by growing them in the presence of increased concentrations of hygromycin have been unsuccessful. Several reports of generating ES cells deficient for a particular gene have been described for selecting for gene conversion events resulting in doubling the number of genomic copies of the neomycin resistance gene (Mortensen et al., 1992), but none so far for the hygromycin resistance gene. This could be due to the different activities of the two antibiotics. Selection of cells in the presence of even higher amounts of hygromycin may allow for the isolation of the desired cells. An even more efficient way to generate these cells may be to electroporate the *p53* heterozygous cells with a *p53* targeting vector carrying a puromycin resistance gene.

Once *Rb-/-;p53-/- ROSA-26* containing ES cells have been generated they can be used to analyze contribution to tissues on a cellular basis. A comparison of the contribution of these cells to tissues relative to *Rb*-deficient *ROSA-26* cells alone may yield important insights into how *Rb*-deficient cells are eliminated by *p53*-dependent apoptosis in different tissues.

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CHAPTER 8

Summary And Implications

As discussed in Chapter 2, a great deal is known about how pRB acts in tissue culture. In addition, many studies have implicated loss of function mutations in *RB* with the development of human cancer. Despite this large collection of data, until recently relatively little was known about the normal role of *Rb* in development. In Chapter 2, the development of techniques which made the creation of mice with germline mutations in *Rb* possible was reviewed. This has allowed several researchers, including myself, to begin to address the physiological role of *Rb* (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992).

The role of *Rb* in development

The creation of *Rb*-deficient mouse strains unexpectedly revealed that *Rb* was not necessary for murine development up to day 13.5 of gestation. This was surprising based on the fact that *Rb* appears to be an important regulator of cell cycle control in almost all cells examined. In addition, higher vertebrates have evolved a complex regulatory network to control the activity of the *Rb* protein through its phosphorylation status (see Figure 2-3). In the case of the INK4/Cyclin D/cdk4 or 6 network, it appears that the only important target relative to cell cycle control may be the *Rb* protein (Guan et al., 1994; Hirai et al., 1995; Lukas et al., 1994; Lukas et al., 1995; Medema et al., 1995). How then do mice completely deficient for *Rb* function progress to such an advanced developmental state? While answering this question has not been the main focus of this work, data presented in Appendices C and E address this question. It appears, at least at some level, that the related *Rb*-family proteins p107 and p130 may play a role in allowing *Rb*-deficient embryos to progress to 13.5 days of development. The generation of animals deficient for both *Rb* and *p107* (Appendix C) as well as animals deficient for both *Rb* and *p130* (G. Mulligan and Tyler Jacks, unpublished) has shown that they fail in development earlier than *Rb*-deficient animals alone, while animals deficient for either *p107* or *p130* fail to show any significant developmental abnormalities (Cobrinik et al., 1996). A rigorous assessment of the molecular basis for this observation will need to be performed to prove there is functional compensation during development for loss of *Rb*-function.

Another related question would be how well animals with an impaired ability to inactivate pRb progress through development. Perhaps the constitutive expression of members of the INK4 family members or the creation of mice deficient in all three cyclin D genes would block development early in gestation. Such experiments may reveal that while the lack of *Rb* function is not compatible with late stages of development, the ability to properly inactivate pRb is required at a very early stage.

While the analysis of *Rb*-deficient mice reveals developmental requirements for *Rb* in hematopoiesis and neurogenesis, mutations in the genes whose products have been shown to regulate pRB suggest that pRB control is important for the normal development of other tissues. As previously discussed, D type cyclins and the INK4 family of kinase inhibitors (which specifically inhibit the actions of D type cyclin-associated cdks) appear to regulate the cell cycle exclusively through their control of pRB (or perhaps pRB-family protein) phosphorylation status. Thus, any defects noted in mice with targeted mutations in these genes may reflect a developmental requirement for proper pRB regulation. The phenotypes of mice deficient for *cyclin D1 were* reviewed in Chapter 2. Briefly, *Cyclin D1*-deficient mice fail to thrive neonatally and exhibit defects in retinal differentiation and mammary development (Sicinsky et al., 1995). The creation of mice deficient for the INK4 protein $p16^{MTS1}$ has also been reported (Serrano et al., 1996). These mice are developmentally normal but are susceptible to the development of sarcomas and lymphomas.

In addition to these mutations in the afferent pathway, mice with targeted gene disruptions in effectors downstream of pRB activity are beginning to be created and analyzed. The first example of such is the creation of mice deficient for the *E2F-1* gene (Field et al., 1996; Yamasaki et al., 1996). Since E2F-1 activity is thought to be regulated by its binding to pRB (and not by association with p107 or p130) defects noted in these mice may suggest additional roles for *Rb* in development. The analysis of these mice revealed that loss of *E2F-1* resulted in tissue atrophy in

some cases. Such a phenotype would be expected based on the idea that *E2F-1* was an oncogene. However, the analysis also revealed that *E2F-1* had properties consistent with it acting as a tumor suppressor gene in some tissues. Perhaps this should change how we view the relevance of the E2F interaction with pRB. The interpretation of the interaction has mainly been that pRB simply inhibited the activity of E2F until pRB was phosphorylated. The release of free E2F would then drive cells into the cell cycle. This data along with several other reports (Weintraub et al., 1995; Weintraub et al., 1992) suggests that E2F facilitates pRB-associated promoter repression. Thus, the interaction between pRB and E2F-1 has regulatory significance for both members of the complex. Whether this model is also operative with other E2F:pRB family member interactions remains to be determined.

The availability of mouse strains with mutations in many of the relevant regulatory and effector genes of the pRB, p107, and p130 pathways should allow significant insight into these and other questions in the future. The generation of mice with mutations in other INK4, cyclin, and E2F genes will undoubtedly follow.

The role of *Rb* in tumor suppression

The major focus of my work has been on using the *Rb*-deficient mice to gain a better understanding of the role of *Rb* in tumor suppression. The tumor predisposition of *Rb* heterozygous mice and children constitutionally heterozygous for an *RB* mutation, while different (discussed below), is in both cases relatively limited. This despite the fact that, at least in humans, *RB* mutations have been observed in many sporadically occurring tumors and alterations in either *Rb* (reviewed in Chapter 2) or the *Rb* regulatory pathway (p16, Cyclin D, cdk4) have been estimated to occur in 80-90% of human tumors (Ed Harlow, personal communication). Why are constitutionally heterozygous mice only predisposed to pituitary and thyroid tumors and humans only retinoblastoma and osteosarcoma?

One possible explanation for this could have simply been that in most tissues a very small window of time exists during which loss of the wild type allele of *Rb* would initiate the process of tumorigenesis. Loss of *Rb* function during any other time would not result in a growth advantage for cells in those tissues. To address this question, we created chimeric mice whose adult tissues were composed of significant numbers of *Rb*-deficient cells (Chapter 5). While these animals lived only to the age of 5 months, they did not exhibit widespread tumorigenesis. The only noticeable neoplasia observed besides intermediate lobe pituitary tumors was in the adrenal medulla, where hyperplastic growths histologically similar to pheochromocytomas were seen. This implies that a small temporal frame in which loss of *Rb* function would induce tumorigenesis could not explain the limited tumor spectrum in constitutionally heterozygous mice.

Another explanation for the limited tumorigenesis seen in *Rb* heterozygous mice was that an accumulation of other mutations was necessary to uncover a carcinogenic predisposition of *Rb*-deficient tissues. The work presented in Chapter 4 shows this to be the case in some instances. The observed cooperativity between *Rb* and *p53* mutations in murine tumorigenesis and the mechanism of suppression of tumors of *Rb*-deficient cells by *p53* has very important implications in human tumors. This is reinforced by the effectiveness of treatment strategies for *Rb*-deficient tumors. Retinoblastomas, which retain wild-type *p53* activity (S. Friend, personal communication), are among the most highly treatable malignancy known (Dryja, 1989). Survival rates of this tumor range up to 95% (S. Mukai, personal communication). Unfortunately, retinoblastoma may be the exception rather than the rule when it comes to the treatment of *Rb* deficient tumors. Almost every other tumor type in humans which has been associated with *Rb* deficiency also has been associated with *p53* deficiency (see Chapter 4). It may be that *p53*-deficiency is a necessary corequisite for tumorigenesis in *Rb*-deficient cells in the majority of tissues. As *p53* deficiency has been associated with a poor prognosis in terms of response to treatment in many human tumors (Bardeesy et al., 1994; Horio et al., 1993; Lowe et al., 1994a; Lowe et al., 1994b; Thorlacius et al., 1993), it may explain why many of the sporadic cancers associated with *Rb* deficiency (for example pancreatic, breast, and lung) respond very poorly to

treatment for example see. The fact that genetic alterations compromising the *Rb* pathway have been estimated to occur in 80-90% of human tumors (Ed Harlow, personal communication) and *p53*-deficiency has been implicated in over 50% of human tumors (Greenblatt et al., 1994) reinforces the importance of understanding the cooperative tumorigenesis observed in more detail. One could envision one day that a highly effective treatment strategy for *Rb*-deficient tumors would be to reintroduce *p53* function (after *p53* functions are better understood) since *Rb*-deficiency has been shown to result in *p53*-dependent apoptosis in several contexts.

A question which stems from the observation of cooperative tumorigenesis between *Rb* and *p53* and the suggestion that *Rb*-deficiency can result in *p53*-dependent apoptosis in some tissues is what is p53 sensing that causes it to induce apoptosis. Several observations must be considered when assessing this question. One is that *Rb*-deficiency alone is clearly not the signal for the apoptotic induction. Many cell types in the *Rb*-deficient embryos and in the *Rb*-DKO chimeras are not susceptible to apoptosis (although this observation in the chimeras is complicated by potential non-cell autonomous effects from the presence of wild-type cells). In addition, work in the *Rb*-DKO chimeras clearly shows that some of the defects seen in *Rb*-deficient embryos can be rescued by the presence of wild-type cells. Finally, not all forms of apoptosis associated with *Rb* -deficiency are dependent on *p53*. Examples of this include *p53*-independent apoptosis in 16.5 day lens fiber cells expressing E7 and the fact that the cell death in the dorsal root ganglia of *Rb*-deficient embryos is still observed in embryos deficient for both *Rb* and *p53* (Macleod et al., 1996). Interestingly, death in the central nervous system of *Rb*-deficient embryos is *p53*-dependent (Macleod et al., 1996).

Work on several *Rb*-deficient cell types suggests that most, if not all *Rb*-deficient cells, have intrinsic alterations in their cell cycle machinery that can be observed under specific circumstances. For example, *Rb*-deficient fibroblasts grown in tissue culture and neurons in *Rb*-deficient embryos and *Rb*-DKO chimeras express constitutively high levels of cyclin E and p21 (Herrera et al., 1996 and Chapter 6). Furthermore, fibroblasts induced to differentiate into myotubes by the

introduction of *myoD* can be stimulated to reenter the cell cycle by exposure to high levels of serum (Novitch et al., 1996; Schneider et al., 1994). This phenomenon is not seen in wild-type fibroblasts and suggests that some component of the cell cycle machinery involved in maintaining or inducing terminal differentiation is abnormal.

A model which can be invoked to explain the fact that *Rb*-deficiency does not lead to apoptosis in more cell types despite the fact that most may be intrinsically abnormal is that a second signal must be present (or absent). One of the best candidates for such a signal would be a survival factor secreted into the cellular environment. The corequirement for the absence of a survival factor with *Rb*-deficiency for apoptotic induction could explain why apoptosis in several *Rb*-deficient embryonic cell types appears to inhibited when these cells are present with wild-type cells in the *Rb*-DKO chimeras. Such a factor could underlie the observed non-cell autonomous phenotypes seen in *Rb*-deficient embryos.

Other reflections on this work

What other important (more general) observations have come out of the research described here? One aspect reinforced by my work can simply be stated that a mouse is not a human being. While that may sound relatively obvious, too often those of us in the mouse "knock-out" field lose track of that point. Because of the incredibly conserved nature of signaling pathways throughout phylogeny, many of us expected that just because the signaling pathways were conserved they were effecting the same endpoint in all organisms. While some murine models of tumor suppressor gene mutations and other gene mutations have similar phenotypes to their human counterparts (for example *Apc* (Dove et al., 1994; Moser et al., 1990) and *p53* (see Appendix A)), many others do not. *Rb*-deficient mice do not develop retinoblastoma. *p16*-deficient mice do not develop melanoma (Serrano et al., 1996). *Nf-1* heterozygous mice are not susceptible to neurofibromatosis (Jacks et al., 1994). This is the case with many other gene knockouts (see Table 2-1). Perhaps some aspects of the artificially controlled laboratory environment are part of

the explanation, but they are unlikely to explain everything. Understanding these species-specific differences will be important in improving the use of these mice to model the human condition associated with deficiencies in these genes and may even enable us to gain further insight into the molecular roles of these genes in cell growth control.

Finally, this work has pointed out another obvious observation overlooked by many in the mouse field which is that cells within an organism are in most cases not isolated entities. Perhaps because molecular biology is so reductionist in its nature, many of us expect that when we create mice deficient for a particular gene that we will find a very clean, easily studied phenotype in these mice. In the case of *Rb* this is clearly not the case. The work presented in Chapter 5 and 6 clearly illustrates that many aspects of the *Rb*-deficient phenotype result from non-cell autonomous defects or at least can be rescued by the presence of wild type cells in the context of a chimera. Specific cells may have paracrine, autocrine, and endocrine functions and a defect in a particular cell type may create several secondary defects. Future work on the *Rb*-deficient mice and the *Rb*-DKO chimeras will undoubtedly shed light on the relevant cellular interactions and secreted factors. This will have important implications in better understanding development and even tumorigenesis in general.

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APPENDIX A

Analysis of the Effects of *p53*-Deficiency on Murine Tumorigenesis and Development

Notes of Attribution

The murine strains described in this work were created by Tyler Jacks, with assistance from Earlene Schmitt and Schlomit Halachmi, while he was a post-doctoral fellow in the Weinberg laboratory. Lee Remington and I performed all the necropsies of affected *p53*-mutant animals. Lee performed the majority of the Southern blot analysis, while I analyzed all the resulting pathology slides with Rod Bronson at Tufts University. The initial analysis of exencephaly (incidence and pathology) in this strain was performed by Valerie (Tan) Sah, an undergraduate whose research I supervised, and myself. Laura Attardi and George Mulligan later analyzed the expression of *p53* and the levels of apoptosis in the *p53*-deficient embryos.

The *p53* tumor suppressor gene is one of the most frequently mutated genes identified in human tumors (Greenblatt et al., 1994; Harris and Hollstein, 1993; Levine, 1992). In order to establish systems to study its function, several groups created strains deficient in *p53*. Analysis of these strains by all four groups resulted in similar conclusions (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994; Tsukada et al., 1994). The majority of animals homozygous for the mutation die in the first six months of life due to the development of one or more aggressive tumors; primarily T-cell lymphomas or sarcomas. Mice heterozygous for the mutation begin to die around one year of age and develop a wide spectrum of tumor types, with an enrichment for sarcoma-type tumors. Thus, the heterozygotes serve as a good model for Li-Fraumeni syndrome (Malkin, 1993; Malkin et al., 1990). This human disorder is characterized by susceptibility to several tumor types relatively early in life in patients who carry a germline mutation in one *p53* allele.

In addition to its effects on tumorigenesis in adult mice, we have found a small, but significant, percentage of *p53*-deficient female embryos do not survive until adulthood. These mice die shortly after birth from exencephaly, a result of defective neural tube closure in the developing midbrain. Interestingly, this phenotype is entirely female specific. The reports of both the tumor studies and the exencephaly are included below.

Tumor spectrum analysis in *p53*-mutant mice

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Background: The *p53* tumor suppressor gene is mutated in a large percentage of human malignancies, including tumors of the colon, breast, lung and brain. Individuals who inherit one mutant allele of *p53* are susceptible to a wide range of tumor types. The gene encodes a transcriptional regulator that may function in the cellular response to DNA damage. The construction of mouse strains carrying germline mutations of *p53* facilitates analysis of the function of *p53* in normal cells and tumorigenesis.

Results: In order to study the effects of *p53* mutation *in vivo*, we have constructed a mouse strain carrying a germline disruption of the gene. This mutation removes approximately 40 % of the coding capacity of *p53* and completely eliminates synthesis of p53 protein. As observed previously for a different germline mutation of *p53*, animals homozygous for this *p53* deletion mutation are viable but highly predisposed to malignancy. Heterozygous animals also have an increased cancer risk, although the distribution of tumor types in these animals differs from that in homozygous mutants. In most cases, tumorigenesis in heterozygous animals is accompanied by loss of the wild-type *p53* allele.

Conclusion: We reaffirm that *p53* function is not required for normal mouse development and conclude that *p53* status can strongly influence tumor latency and tissue distribution.

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Background

The *p53* tumor suppressor gene appears to be a critical regulator of normal cell proliferation. This is indicated by the fact that mutant alleles of this gene are found in a majority of human tumors, involving a wide range of tumor types [1]. In addition to the frequent somatic mutation of *p53* in sporadic cancer, germline mutation of one allele of this gene in humans causes an inborn predisposition to cancer known as Li–Fraumeni syndrome [2]. Individuals with Li–Fraumeni syndrome are highly prone to the development of sarcomas and a variety of other tumor types, including carcinomas of the breast and brain [3].

Although the precise mechanism(s) by which *p53* normally suppresses tumor formation is not known, recent experiments have implicated the gene in controlling cell-cycle arrest in response to damage to the genome resulting from radiation [4,5] and gene amplification [6,7]. Moreover, following certain stimuli, *p53* function is required for some cells to undergo programmed cell death (apoptosis) [8,9]. The *p53* gene encodes a DNA-binding protein [10] that can have both positive and negative effects on transcription [11–16].

Recently, Donehower *et al.* [17] have utilized the technique of gene targeting in murine embryonic stem (ES) cells to construct a mouse strain carrying a germline mutation in the *p53* gene. These authors reported the surprising observation that mice homozygous for this mutation are viable. Thus, despite its clear importance in suppressing the transformation of a number of cell types, *p53* is not essential for normal development of the mouse. However, the presumed role of *p53* in tumor suppression was supported by the observation that homozygous mutant animals were highly prone to malignancy in the first months after birth [17]. These authors also reported tumors in a small number of animals heterozygous for the *p53* mutation. Thus, as is the case with humans suffering from Li–Fraumeni syndrome, a germline mutation of *p53* in the mouse can lead to increased cancer susceptibility.

In an effort to confirm and extend the observations of Donehower *et al.* [17], we have constructed a mouse strain carrying a *p53* mutant allele that is different from the one described by them. This mutation, which deletes approximately 40 % of the *p53* coding region, completely blocks production of p53 protein. Consistent with the earlier report [17], homozygosity for this *p53* deletion mutation is compatible with normal murine development. We have also studied the kinetics and tissue distribution of tumor formation in animals homozygous and heterozygous for this *p53* mutation. The data demonstrate that the heterozygous tumor phenotype closely resembles that seen in Li–Fraumeni syndrome and differs from that observed in the homozygous mutant animals. The tissue distribution of tumors in heterozygous mice may reflect the somatic mutation rate in different tissues and cell types.

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Results

p53 gene targeting

One allele of the *p53* gene was disrupted in D3 ES cells using the targeting vector *p53*KO shown in Figure 1. The neomycin resistance gene (*neo*) in this vector is flanked by fragments of *p53* totaling 8 kilobases (kb); *neo* replaces approximately 40 % of the *p53* coding sequences, beginning within exon 2 (upstream of the initiator codon AUG) and extending into intron 6. The HSV-TK gene follows the *p53* sequences to allow negative selection using the protocol of Mansour *et al.* [18]. *p53*KO was introduced into D3 cells by electroporation and, following selection in G418 and gancyclovir (GANC), cell clones were screened by Southern blotting of their DNA.

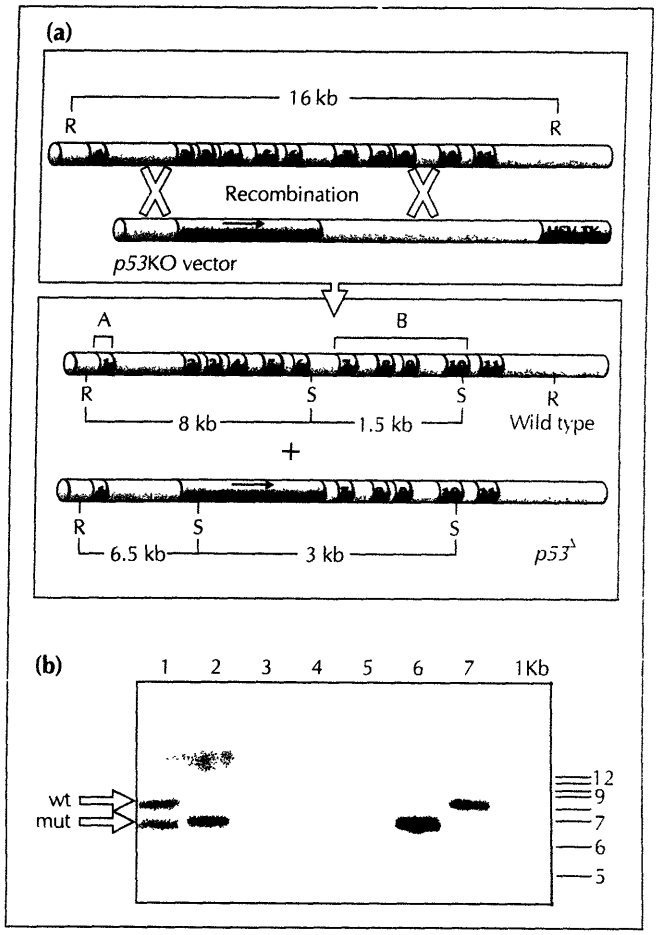

Fig. 1. *p53* gene targeting. **(a)** Scheme for targeting one allele of the murine *p53* gene in ES cells. Homologous recombination between the *p53*KO targeting vector and one allele of the endogenous *p53* gene results in the replacement of *p53* coding sequences between exons 2 and 7 with the *neo* gene expression cassette and the formation of the $p53^{\Delta}$ mutant allele. ES cell clones were screened by Southern blotting of genomic DNA digested with *EcoR*I (R) plus *Stu*I (S) and hybridized to probe derived from *p53* exon 1 (A), which is not present in *p53*KO. The structure of the mutant allele in putative heterozygous ES-cell clones was confirmed using a 3′ cDNA probe (B), covering exons 7–10. Details of the construction of *p53*KO are described in Materials and methods. **(b)** Genotypic analysis of offspring from a *p53* heterozygous cross. Tail DNA was isolated from offspring at weaning and examined by Southern blotting as described above using probe A. This probe hybridizes to an 8.0 kb R–S fragment from the wild-type allele (wt) and a 6.5 kb R–S fragment from the $p53^{\Delta}$ allele (mut). Animals 2 and 6 are $p53^{\Delta/\Delta}$, animal 7 is $p53^{+/+}$, and animals 1, 3 ,4, and 5 are $p53^{+/\Delta}$. Molecular weight standards are shown (lane 1Kb), along with their sizes (in kb) at right.

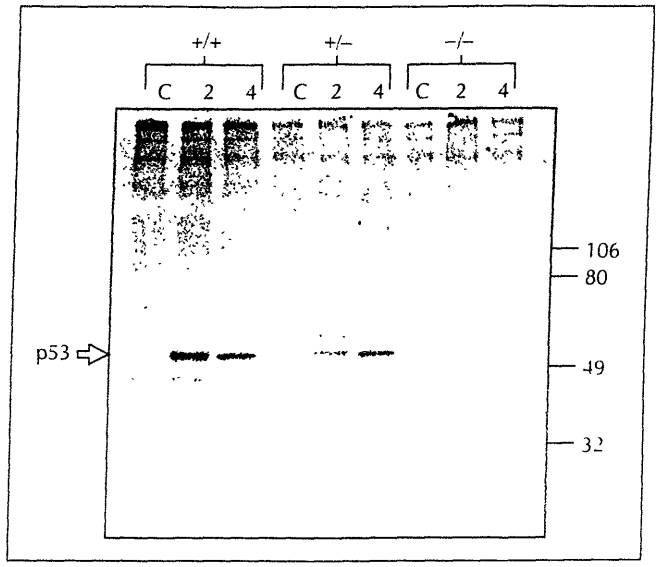

Fig. 2. Immunoprecipitation of p53 protein. Fibroblasts isolated from wild-type (+/+), heterozygous (+/-), and homozygous mutant (-/-) embryos were labeled with a [^{35}S] methionine and [^{35}S] cysteine, and whole cell lysates precipitated with negative control (C) or two p53-specific antisera: pAb248 (2) and PAb421 (4). Position of molecular size markers are shown (in kD) on the right and the expected position of p53 protein on the left. Note the absence of a specific signal from -/- cells and that the signal from the +/- cells is reduced compared with +/+ cells.

A probe derived from exon 1 of *p53* (and not present in the *p53*KO targeting vector) hybridizes to an *EcoR*I to *Stu*I fragment of approximately 8 kb from the wild-type *p53* allele, whereas the mutant allele created by homologous recombination between *p53*KO and the endogenous *p53* gene yields a 6.5 kb fragment (Fig. 1). Of 200 G418/GANC-resistant ES cell clones screened, 8 were found to be heterozygous for the *p53* mutation (data not shown). The calculated enrichment from the HSV-TK counterselection [18] was six-fold, indicating an overall targeting efficiency of 1/150 per *neo*-resistant clone. Because this mutation results in the deletion of a significant fraction of the *p53* coding sequence, we have designated this allele $p53^{\Delta}$.

p53-mutant mice

Injection of three independent heterozygous ES cell clones into wild-type C57BL/6 blastocysts produced male chimeras that were able to transmit the $p53^{\Delta}$ mutation upon breeding to C57BL/6 females. Male and female heterozygous mice were then intercrossed to determine the viability of homozygous mutant animals. Of the first 422 offspring of heterozygous crosses, 120 were $p53^{+/+}$, 232 were $p53^{+/\Delta}$ and 70 were $p53^{\Delta/\Delta}$. A representative Southern blot of tail DNA from one litter of mice is shown in Figure 1.

Throughout their first few weeks, the gross appearance of the $p53^{\Delta/\Delta}$ animals was normal, and they were

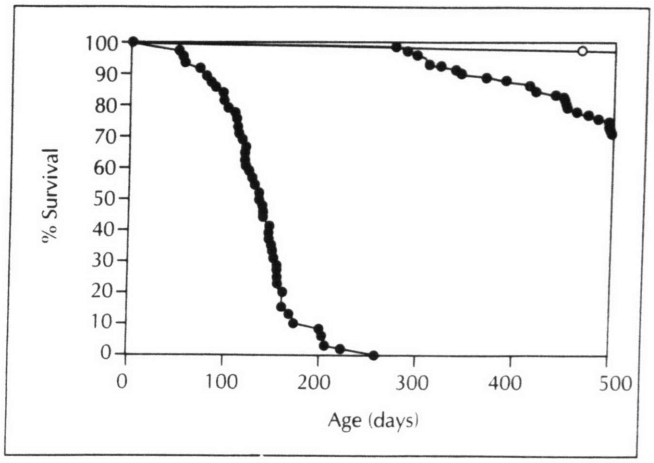

Fig. 3. Effects of $p53^{\Delta}$ mutation on survival. Viability of 34 wild-type animals (white circles), 100 heterozygotes (blue circles), and 49 homozygous mutants (red circles) was monitored for a total of 500 days. Points represent animals that had died or had to be sacrificed owing to ill health. The single wild-type animal that died during this period had developed a tumor. Tumors were also observed in 17 of 24 heterozygotes and 41 of 49 homozygotes examined by necropsy (see text).

fertile. Thus, the homozygous mutation of *p53* is compatible with normal embryonic and postnatal development. These results are consistent with the earlier report of Donehower *et al.* [17] describing a different germline mutation of *p53* . In our experiments, the yield of $p53^{\Delta/\Delta}$ offspring from heterozygous crosses has been only 16.6 % (70/422), rather than the expected 25 %. This may be the result of the death of a fraction of homozygotes either during embryogenesis or after birth but before weaning.

Analysis of p53 protein

The effect of the $p53^{\Delta}$ mutation on the synthesis of p53 protein was examined using fibroblasts isolated from $p53^{+/+}$, $p53^{+/\Delta}$, and $p53^{\Delta/\Delta}$ embryos. Early passage cells were labeled with [^{35}] methionine and [^{35}S] cysteine, and whole-cell lysates were precipitated with two p53-specific monoclonal antibodies. The antibodies PAb421 and PAb248, which recognize epitopes in the amino and carboxyl termini of the protein, respectively [19], precipitate full-length p53 from wild-type and heterozygous cells, whereas neither antibody detects a specific protein product in homozygous mutant fibroblasts (Fig. 2). Thus, the $p53^{\Delta}$ mutation eliminates the production of full-length p53, and the mutant allele does not appear to yield any truncated proteins that accumulate in cells. In addition, we have consistently observed that the steady-state level of p53 protein in heterozygous fibroblasts is approximately half of that in wild-type cells (Fig. 2).

$p53^{+/\Delta}$ mice as a model for Li–Fraumeni syndrome

In humans, inheritance of one mutant allele of *p53* causes a broad-based cancer predisposition known as Li–Fraumeni syndrome [2]. The predominant tumor types in humans who are heterozygous for a *p53* mutation are sarcomas and carcinomas of the breast and brain [3]. We have closely monitored $p53^{+/\Delta}$ animals for signs of malignancy. As shown in Figure 3, heterozygosity for the $p53^{\Delta}$ mutation does not affect viability until the mice are approximately nine months of age. By 17 months, 28 % (28/100) of the heterozygous animals had died or had to be sacrificed because of ill health. Necropsy revealed obvious tumors in 17 of 24 of these animals.

Figure 4 shows the distribution of tumors observed in heterozygous animals. The most common tumor types were sarcoma (including fibrosarcoma, osteosarcoma, rhabdomyosarcoma, hemangiosarcoma, anaplastic sarcoma and lyomyosarcoma) in 57 % (25/44) and lymphomas in 25 % (11/44) of animals. We have also observed one brain ependymoma, one hair matrix tumor, five lung adenocarcinomas, and two hepatomas in this group of heterozygous animals. Pathological sections of a typical osteosarcoma, a rhabdomyosarcoma, and a hair matrix tumor are shown in Figure 5.

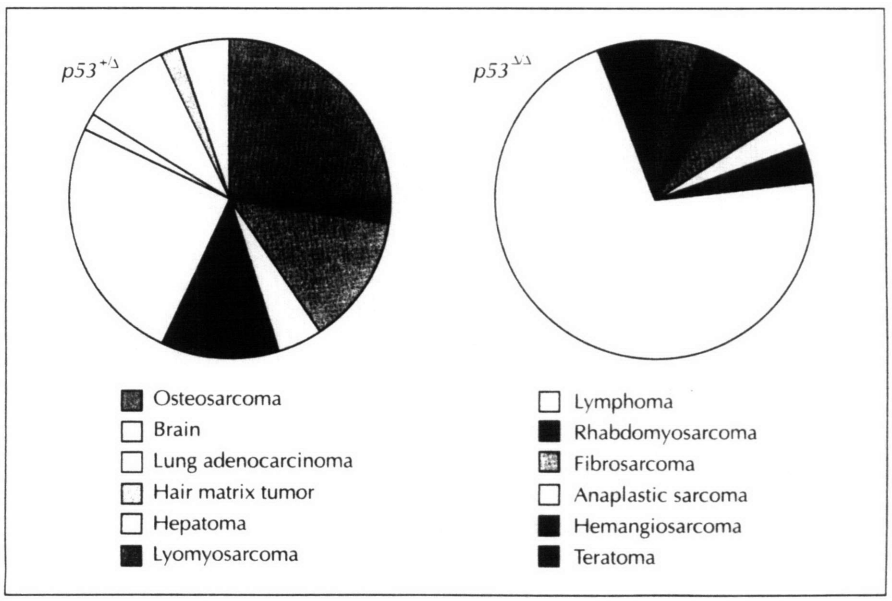

Fig. 4. Tumor distribution in heterozygous and homozygous mutant animals. Pie charts show the relative frequency of tumor types observed in $p53^{+/\Delta}$ and $p53^{\Delta/\Delta}$ mice. Frequencies determined from 44 total tumors in heterozygotes and 56 total tumors in homozygotes. Note that the sarcoma is the most common tumor type in heterozygotes, whereas lymphomas are most common in homozygotes.

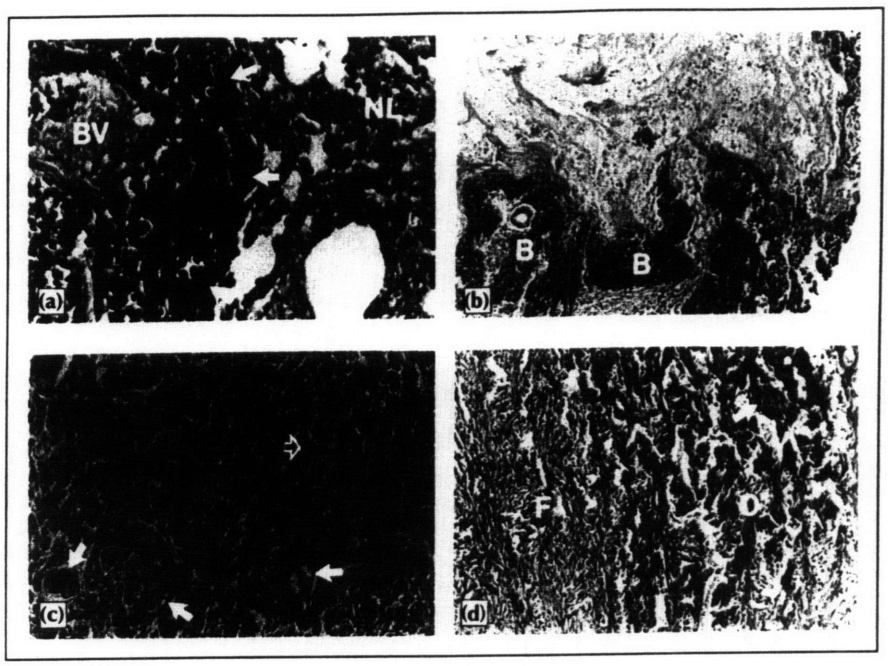

Fig. 5. Histopathology of representative tumors. **(a)** Thymic lymphoma from a $p53^{\Delta/\Delta}$ animal with tumor cells surrounding a blood vessel (BV). Arrows delineate the border of the tumor and normal lung (NL), which may contain infiltrating tumor cells. **(b)** Hair matrix tumor from a $p53^{+/\Delta}$ animal consisting of regions of transformed basal cells (B) and (above) exfoliating squamous cells, ghost cells, and keratin. **(c)** Rhabdomyosarcoma isolated from a heterozygous animal showing tumor giant cells (filled arrows) and a characteristic strap cell (unfilled arrow). **(d)** Osteosarcoma from a $p53^{+/\Delta}$ animal. Tumor consists of both fibroblastic (F) and osteoblastic (O) regions; a characteristic bone spicule is indicated (arrow). Magnification: panels **(a)** and **(c)**, 300X; panels **(b)** and **(d)**, 75X.

We conclude that the tumor spectrum observed in *p53* heterozygous mice is similar (although not identical) to that seen in humans with Li–Fraumeni syndrome.

Loss of heterozygosity

The vast majority of *p53* mutations in sporadic cancers in humans are base substitutions that cause missense changes in the coding region of the gene [1]. Many of the resulting mutant p53 proteins have increased half-lives and are thought to interfere with the function of wild-type p53 protein expressed in the same cell [20]. In addition to their apparent role in eliminating residual wild-type *p53* activity in the cell, such mutant p53 proteins may act positively by contributing to cell proliferation even in the absence of any wild-type p53 protein [21,22]. Therefore, it was of interest to examine the *p53* status in the tumors of $p53^{+/\Delta}$ animals. In particular, we wished to determine if the wild-type allele of *p53* was simply lost during tumorigenesis or whether it had acquired an 'activating' mutation resembling those present in many human tumor DNAs.

The *p53* status of tumor DNA was analyzed using Southern blotting (Fig. 6) or the polymerase chain reaction (PCR; data not shown). In 9/12 tumors tested, the wild-type allele of *p53* was either completely absent or clearly under-represented. Most of the tumor DNA samples did show a residual signal from the wild-type allele (Fig. 6), which we assume results from contamination of the tumor preparation with surrounding normal tissue.

These data indicate that *p53* function is eliminated during the development of most, if not all, tumors in the $p53^{+/\Delta}$ animals. Furthermore, the loss of the wild-type allele typically occurred via a chromosomal mechanism (that is, large deletion, mitotic recombination or chromosomal nondisjunction) rather than by point mutation. In the minority of tumors that did not show loss of the wild-type *p53* allele, the gene was either not mutated or had sustained a point mutation or other subtle lesion that would not have been detected by our methods. We conclude that the creation of a point-mutated allele of *p53* is not a prerequisite for tumorigenesis in these animals.

Accelerated tumorigenesis in $p53^{\Delta/\Delta}$ mice

Although initially healthy, mice that are homozygous for the $p53^{\Delta}$ mutation are highly predisposed to malignancy. As shown in Figure 3, homozygotes have a greatly accelerated rate of tumorigenesis compared with heterozygous or wild-type mice. Of the first 49 homozygotes screened, 46 died by six months of age. By nine months, all animals had died or had to be sacrificed. Necropsy revealed obvious tumors in 41 of the 49 animals. Six mice were discovered to have two distinct tumors, often a lymphoma with either a sarcoma or a teratoma.

Whereas mice heterozygous for the $p53^{\Delta}$ mutation were most prone to developing sarcoma, the predominant tumor type in the $p53^{\Delta/\Delta}$ animals was lymphoma at 71 % (44/56), usually affecting the thymus (Fig. 4). A section through a representative thymic lymphoma is shown in Figure 5(a). Homozygous mutant animals also developed other tumor types, including osteosarcoma, rhabdomyosarcoma, hemangiosarcoma and teratoma.

In order to determine the cell type of origin of the thymic tumors, we screened cells isolated from four tumor samples by flow cytometry using a number of T-cell-specific markers. All four tumors stained positively for both CD4 and CD8 (data not shown), indicating that they arose from the transformation of relatively immature thymocytes [23]. We have successfully adapted CD4/CD8 double-positive cells isolated from several of the thymic lymphomas to growth in tissue culture, which will facilitate further analysis.

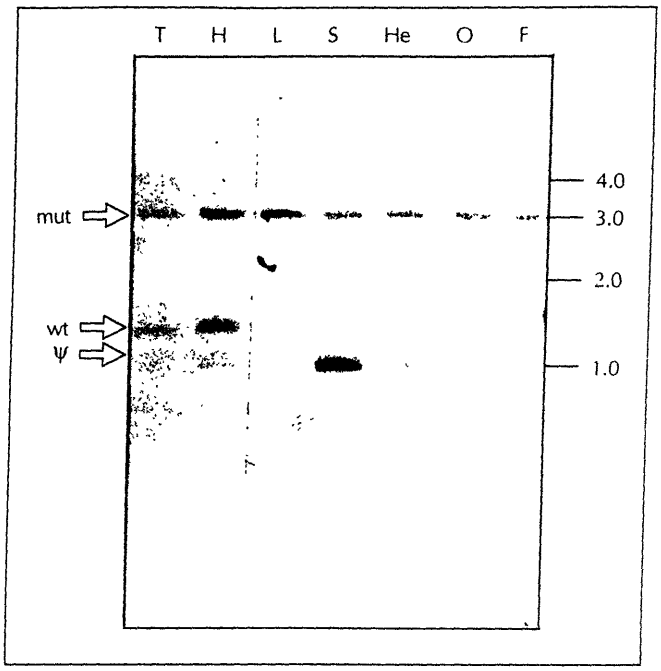

Fig. 6. Loss of heterozygosity. Southern blot analysis of DNA isolated from tumors of $p53^{+/\Delta}$ mice. DNA was digested with *EcoRI* and *StuI* and hybridized with probe B shown in Figure 1. Tail DNA (T) from a heterozygous animal shows three bands, which are derived from the wild-type *p53* allele (wt), the $p53^{\Delta}$ allele (mut) and the *p53* pseudogene (ψ). Of the six tumor samples shown, five show a greatly reduced signal from the wild-type allele, consistent with its loss during tumorigenesis. One tumor (H) has retained the germline pattern. H, hepatoma; L, lymphoma; S, anaplastic sarcoma; He, hemangiosarcoma; O, osteosarcoma; F, fibrosarcoma.

Discussion

The method of gene targeting in mouse ES cells [24] has allowed the construction of a large number of novel mouse strains that can be used to study diverse areas of biology, including development, immunology, human genetic disease and cancer. Here, we have described the properties of a mouse strain carrying a targeted deletion mutation in the *p53* tumor suppressor gene. Given the importance of *p53* mutations in sporadic and familial forms of cancer, this strain and analogous strains constructed by others represent a valuable resource for cancer research.

Donehower *et al.* [17] first reported the unexpected observation that mice homozygous for a particular *p53* mutation were viable. This mutation replaces a part of *p53* intron 4 and the 5' portion of exon 5 with a *neo* expression cassette [17]. We have constructed a second mutant allele of *p53*, one that clearly blocks production of p53 protein, and we find that animals homozygous for this mutation also survive gestation normally. Therefore, although *p53* is mutated in a large fraction of human malignancies [1], the function of this gene is dispensable for normal development, at least in the mouse. Homozygous mutant animals are, however, highly predisposed to cancer. All 49 homozygotes followed in this study died by nine months of age, and (with the exception of eight animals on which necropsy could not be performed) all had obvious tumors.

The absence of a developmental defect in *p53*-deficient animals coupled with their greatly increased cancer risk suggests that *p53* might function specifically as a suppressor of tumorigenesis rather than as a general cell-cycle regulator. Consistent with this view, we and others have recently shown that *p53* is required for the normal cellular response to DNA damage, either G1 growth arrest in fibroblasts [5] or apoptosis in thymocytes [8,9]. The failure of *p53*-deficient cells to undergo proper cell-cycle arrest or programmed cell death following DNA damage could result in an increased mutation rate or the inappropriate survival of damaged cells [25]. *p53* is also required for apoptosis in cells expressing the adenovirus *E1A* oncogene ([26] and S. Lowe, T.J., D. Housman and H.E. Ruley, manuscript submitted). Thus, in addition to acting to limit the mutation rate (including the rate of oncogenic mutations), *p53* may also function to eliminate cells that have already undergone oncogenic mutations. Accordingly, cells lacking *p53* function would be expected to be more readily transformed as a result of the abrogation of this death program.

As reported here, the most common tumor observed in *p53*-deficient mice is thymic lymphoma, and in all cases examined the tumor cells were found to express the surface antigens present on immature thymocytes. Given the requirement for *p53* function in radiation-induced apoptosis in this cell type [8,9], the high frequency of thymic lymphoma may result from the failure to eliminate cells that have suffered some form of DNA damage. One potential source of DNA damage during T-cell development is the rearrangement process involving the T-cell receptor (TCR) genes. The state of the TCR genes has not yet been examined in these tumors.

In addition, we have found that mice heterozygous for the $p53^{\Delta}$ mutation are predisposed to cancer. The tumor phenotype of these animals differs from that of homozygotes, both with respect to latency and distribution of tumor types. The longer tumor latency observed in heterozygous mice could be explained by the requirement for heterozygous cells to mutate the wild-type *p53* allele at some point during tumorigenesis. Indeed, three quarters of the tumors from $p53^{+\Delta}$ animals examined here showed loss of the wild-type *p53* allele. This result indicates not only that elimination of *p53* function is selected for during tumorigenesis, but also that the acquisition of a potentially activating point mutation in the remaining *p53* allele is infrequent in this setting. These data are compatible with models in which point-mutated alleles of *p53* act predominantly to inhibit the function of wild-type p53 protein.

Heterozygous animals also differ from homozygotes in the spectrum of tumors to which they are predisposed, with sarcomas being the most common tumor type

observed in heterozygotes rather than the lymphomas of the homozygotes. Given the requirement to mutate the wild-type *p53* allele in heterozygotes, an important factor in determining the tumor spectrum in these animals may be the frequency of this mutational event in different tissues. In particular, the relatively slow elimination of the wild-type *p53* allele would be expected to limit the frequency of thymic lymphoma in heterozygotes, as the number of immature thymocytes drops dramatically following thymic involution at several weeks of age.

The value of animals carrying a germline *p53* mutation extends beyond the analysis of their particular tumor phenotypes. The animals described here have been extremely useful as a source of mutant cells with which to study *p53* function *in vitro* [5,7,9,27]. Also, *p53*-mutant mice can be bred to other cancer-prone mouse strains (for example, other tumor suppressor gene mutants [28–30] or oncogene transgenics [31]) in order to examine the possible cooperative effects of *p53* deficiency and other oncogenic mutations. These animals represent an important tool in the continuing analysis of the role of the *p53* tumor suppressor gene in controlling malignant transformation.

Conclusions

The generation and characterization of *p53*-deficient mouse strains has provided important insights into the role of this gene in growth control and neoplasia. The surprising dispensability of *p53* function for embryonic development demonstrates that *p53* is not required for normal cell growth or differentiation. However, *p53* function is clearly important to the suppression of tumorigenesis, as heterozygous and homozygous mutant animals are highly cancer prone. In addition, *p53* status (wild-type, heterozygous or homozygous mutant) is an important determinant of the latency and tissue distribution of tumors.

Materials and methods

Gene targeting

*p53*KO was constructed by cloning fragments of the murine *p53* gene (isolated from strain BALB/c) [32] into the targeting vector pPNT [33]. The *neo* gene in *p53*KO is flanked by a 3.5 kb fragment extending from the *BamH*I site in intron 1 to the *Nco*I in exon 2 and a 5 kb fragment extending from the *BamH*I in intron 6 to the *EcoR*I downstream of exon 11 [32]. Electroporation of *p53*KO into D3 ES cells [34], subsequent drug selection, and Southern blot analysis were performed as described [28]. The Southern blot probe A (Fig. 1) consists of an *EcoR*I to *Bsm*I fragment of *p53* genomic DNA including exon 1; probe B is a *Sac*II to *Kpn*I fragment of *p53* cDNA which includes exons 7 through 10.

Heterozygous ES cells (derived from strain 129/SV) were injected into C57BL/6 blastocysts essentially as described in [35]. Extensively chimeric males were screened for germline contribution of the mutant cells by breeding to C57BL/6 females. Genotypic analysis was performed by Southern blotting (see above) or PCR. The wild-type *p53* allele was amplified using PCR primers directed against exon 6 (W5': 5'-ACAGCGTGGTGGTACCTTAT-3') and exon 7 (W3': 5'-TATACTCAGAGCCGGCCT-3'), whereas the $p53^{\Delta}$ allele was amplified using a primer directed against *neo* (M5': 5'-CTATCAGGACATAGCGTTGG-3') and W3'. Homozygous offspring were generated initially from heterozygous intercrosses and subsequently from homozygous intercrosses. All of these animals are on a 129/SV x C57BL/6 mixed genetic background.

p53 protein analysis

Immunoprecipitation of p53 protein was performed as described in [7] using early passage fibroblasts from wild-type, heterozygous and homozygous mutant embryos, prepared as described in [36]. Antibody PAb421 was purchased from Oncogene Science. Control antiserum (M73, adenovirus E1A) and PAb248 were generously provided by E. Harlow.

Tumor analysis

Animals that died or were sacrificed were subjected to complete necropsy. Tumor samples were surgically removed and fixed in Bouin's solution. Specimens were processed for histology, embedded in paraffin, sectioned at 6 μ, and stained with hemotoxylin and eosin. Loss of heterozygosity analysis was performed on DNA isolated from fresh tumor samples using either Southern blotting or PCR as described above.

Tumor cells isolated from thymic lymphomas were stained with phycoerythrin-conjugated anti-CD4 and fluorescein isothyocyanate-conjugated anti-CD8 antibodies (anti-L3T4 and anti-Lyt2, Becton Dickinson) and analyzed using a FACStar Plus fluorescence-activated cell sorter (Becton Dickinson).

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A subset of *p53*-deficient embryos exhibit exencephaly

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Defects in neural tube formation are among the most common malformations leading to infant mortality. Although numerous genetic loci appear to contribute to the defects observed in humans and in animal model systems, few of the genes involved have been characterized at the molecular level. Mice lacking the *p53* tumour suppressor gene are predisposed to tumours, but the viability of these animals indicates that p53 function is not essential for embryonic development. Here, we demonstrate that a fraction of p53-deficient embryos in fact do not develop normally. These animals display defects in neural tube closure resulting in an overgrowth of neural tissue in the region of the mid-brain, a condition known as exencephaly.

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The *p53* tumour suppressor gene is frequently mutated in a wide range of human cancers[1]. Recent evidence suggests that the gene is required for G1 growth arrest following DNA damage[2,3] or the induction of apoptosis[4–11], depending on the cell type. Interestingly, mice lacking p53 are viable, although they are prone to developing a variety of tumours within four to six months of age[12–15]. The viability of animals lacking p53 has been taken to indicate that p53 plays no role in normal mouse development.

Malformations of the developing neural tube are common congenital defects, with frequencies worldwide that vary from one to nine per 1,000 total births[16]. Anencephaly, a lethal condition in which the brain tissue is exposed, and spina bifida, a condition involving regionalized disruption of spinal nerve pathways, are the most prevalent forms of neural tube defects (NTD)[17]. The aetiology of these defects is not well defined, but genetic and environmental factors are known to influence NTD in human populations and mouse model systems. Although these and other studies strongly suggest that different inherited factors contribute to NTD, the identity of such loci remains unclear.

Mouse models of NTD resemble to varying degrees the human condition with respect to location and pathology of the defect, female preference, partial penetrance and environmental sensitivity[17,18]. Several mouse mutants exhibit exencephaly, a defect related to anencephaly, in which the brain protrudes above the skull. In the Splotch and curly tail mutants, both exencephaly and spina bifida are observed. The spina bifida observed in 15–20% of curly tail (*ct*) mutant embryos[19] appears to involve a specific proliferative defect in the gut endoderm and notocord[20]. Although the molecular basis for the *ct* mutation remains uncharacterized, a recent study has mapped the gene to mouse chromosome 4 and identified several modifier loci[16]. Despite such evidence that neural tube defects involve multifactorial inheritance, the mechanistic processes disrupted by these or other genetic lesions remain obscure. It is likely that the defects in a variety of systems may lead to abnormalities in the complex process of neurulation

Mice lacking the *p53* tumour suppressor gene are indistinguishable from their wild type littermates except for their dramatically early onset of tumour formation[12,13]. It has been noted, however, that the frequency of recovery of these homozygous mutant animals is slightly less than anticipated[13]. We describe here a developmental analysis demonstrating that a variable percentage of *p53*-deficient animals exhibits mid-brain exencephaly, a neural tube defect that is incompatible with post-natal survival.

Phenotype of *p53*-deficient embryos

Crosses between mice heterozygous for a targeted mutation in exon 5 of *p53* have yielded 23% homozygous mutant offspring (close to the expected mendelian ratio of 25%), leading to the conclusion that p53 function is not necessary for mouse development[12]. However, in our analysis of animals produced from heterozygous intercrosses of mice carrying the $p53^{\Delta}$ mutation on a mixed (129/sv × C57BL/6) genetic background, the frequency of homozygous mutants ($p53^{-/-}$) recovered at weaning was 20%, slightly less than the anticipated value (Table 1). The reduced yield of $p53^{-/-}$ animals from such crosses was more apparent on the inbred 129/sv genetic

Table 1 *p53* genotypes of adult and embryonic mice

Mouse background	Heterozygous–heterozygous crosses			
	$p53^{+/+}$	$p53^{+/-}$	$p53^{-/-}$	$p53^{-/-a}$
Adults				
Mixed	176 (30.2%)	290 (49.7%)	117 (20.1%)	N/A
129/sv	35 (24.3%)	84 (58.3%)	25 (17.4%)	N/A
Embryos[b]				
Mixed	42 (28.8%)	70 (48.0%)	31 (21.2%)	3 (2.1%)
129/sv	7 (21.9%)	19 (59.4%)	4 (12.5%)	2 (6.2%)
		Heterozygous-homozygous crosses		
Embryos[c]				
Mixed	—	215 (56.3%)	153 (40.1%)	14 (3.7%)
129/sv	—	49 (47.1%)	47 (45.2%)	8 (7.7%)

[a]Exencephaly. [b]13.5 gestation days. [c]12.5–18.5 gestation days. Offspring of crosses between $p53^{+/-}$ were weaned and genotyped at three weeks of age. The numbers of animals recovered of each genotype on a mixed or 120/sv inbred background are indicated. Day 13.5 embryos were also analyzed and the occurrence of mutants exhibiting exencephaly is indicated. For each category percentages of the total number of specimens analysed are indicated in parentheses. The lower portion of the table summarizes the analysis of embryos from crosses of $p53^{-/-}$ with $p53^{+/-}$ animals.

background, where just 17% of the offspring were of this genotype (Table 1). These data suggest that a fraction of the p53-deficient animals either do not survive gestation or die sometime after birth prior to weaning. In order to address whether some *p53*-deficient embryos developed abnormally, we examined litters from heterozygous intercrosses at day 13.5 of gestation. Of 34 *p53*-deficient embryos recovered at this stage from the mixed genetic background, 3 (8.8%) displayed abnormal head and brain morphology. Moreover, 14 of 167 (8.4%) $p53^{-/-}$ embryos derived from heterozygous-homozygous crosses on the mixed genetic background had this appearance (Table 1). This defect was characterized by an overgrowth of neural tissue, usually confined to the region of the fore- and mid-brain, but occasionally also affecting the hind-brain (Fig. 1*a*). The neural overgrowth precluded normal formation of the cranium in this region and therefore the brain protruded above the skull, a condition termed exencephaly.

The percentage of exencephalic embryos was higher on the 129/sv inbred genetic background, where 10 of 61 $p53^{-/-}$ embryos (16%) derived from either heterozygous–heterozygous or heterozygous–homozygous crosses exhibited this phenotype consistent with the lower yield of liveborn $p53^{-/-}$ animals on the inbred background (Table 1). None of 402 $p53^{+/+}$ or $p53^{+/-}$ embryos examined from the two genetic backgrounds were exencephalic, nor has exencephaly ever been observed in over 1,000 embryos derived from heterozygous intercrosses of mice with mutations in *Rb* or *Nf1* on these genetic backgrounds[21,22]. Furthermore, this developmental phenotype was due to the absence of p53 function rather than a mutation in some closely linked gene, since we observed exencephaly in two independent lines of *p53*-mutant mice derived from different targeted embryonic stem cell clones[13].

Exencephaly results from the failure of neural tube closure followed by outward expansion of neural tissue via the eversion of the neural plate. As shown in Fig. 1*c* and *d*, the exencephalic $p53^{-/-}$ embryos clearly displayed the persistent opening of the neural tube in the region of the fore- and mid-brain. Histological sections of the $p53^{-/-}$ exencephalic embryos were characterized by absence of bilateral symmetry on the two sides of the forebrain (Fig. 2*a*,*c*,*e*). The surface of the cerebrum was covered by ependymal cells, as if the ventricles were turned inside out. Numerous random invaginations of this surface layer deep into the distorted cerebrum resulted in the formation of many small pseudo-ventricles and rosettes of ependymal cells (Fig. 2*e*). Except for this particular neurological defect, these exencephalic embryos were otherwise histologically normal (Fig. 2*a*), and they were

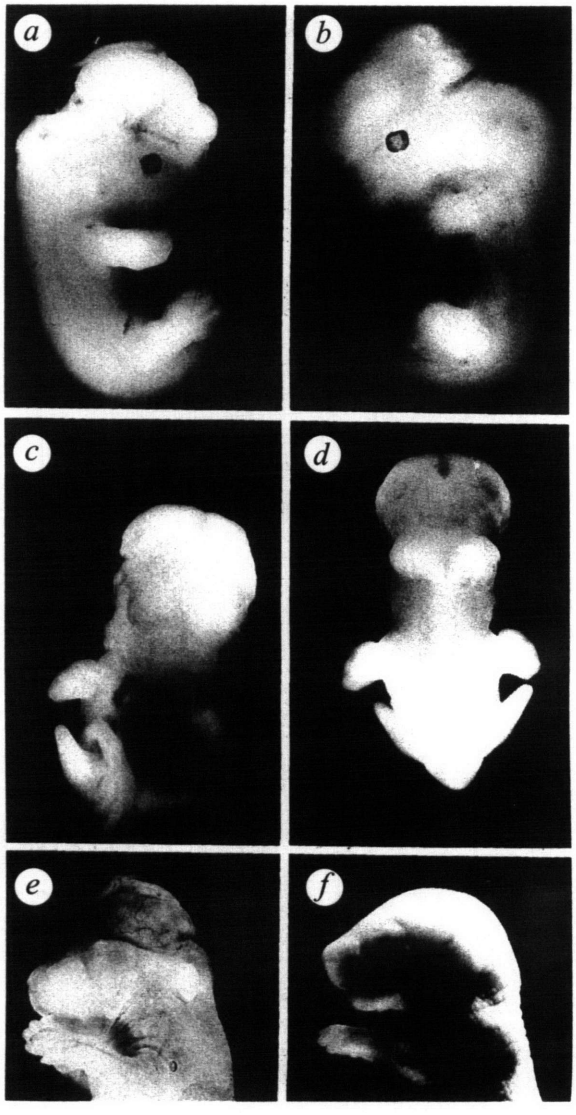


Fig. 1 Whole mount view of $p53^{-/-}$ exencephalic embryos and littermate controls. *a*, Lateral view of 13.5 day exencephalic $p53^{-/-}$ embryo. Note that the skull is open caudally. *b*, Littermate of animal shown in (*a*). The skull covers the brain completely. Note the well-defined lateral ventricle. $p53^{+/+}$, $p53^{+/-}$ and non-exencephalic $p53^{-/-}$ embryos were indistinguishable visually. *c*, Ventral view of a 13.5 day exencephalic $p53^{-/-}$ embryo in which the cerebrum is exposed and the two hemispheres are asymmetric. *d*, Dorsal view of embryo in *c* with exposed mid- and hind-brain. *e*, Lateral view of 18.5 day exencephalic $p53^{-/-}$ embryo. *f*, Normal littermate of animal shown in *e*.

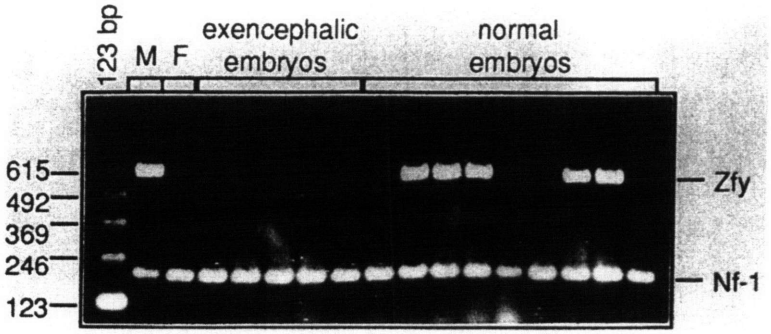


Fig. 3 Sex determination of exencephalic *p53*$^{-/-}$ embryos. DNA samples from control male (M) and female (F) adult mice, embryos of normal appearance, and *p53*$^{-/-}$ exencephalic embryos were subjected to PCR analysis for sex determination. The presence of Y chromosomal DNA in males produced a 610 bp fragment with primers directed against the *Zfy* gene[23]. All five exencephalic embryos shown and 22/22 additional exencephalic embryos lacked the *Zfy* band and were, therefore, female. Primers directed against the autosomal *Nf1* gene[22] were included as a control for the presence for DNA. Sizes (in base pairs) of molecular weight standards (123 bp) are shown at left.

targeted mutation in the *p53* gene (L. Donehower *et al.* pers. comm.). Thus, the absence of p53 function during development in the mouse can lead to a dramatic, and ultimately fatal, embryological abnormality. This phenotype is consistent with the high-level expression of *p53* we and others have seen during early embryogenesis, both in the developing brain and other tissues[26,27]. Additionally, the discovery of a function for *p53* in mouse embryogenesis underscores the fact that tumour suppressor genes are important in normal development as well as tumorigenesis. For example, homozygous inactivation of the *Rb*[21,28,29], *Wt1*[30] and *Nf1*[22,31] tumour suppressor genes all lead to embryonic lethality associated with specific developmental abnormalities.

The developmental phenotype of *p53* mutants exhibits partial penetrance, which varies depending on the genetic background. This finding implies the presence of modifying genes and thus suggests that the process of neural tube closure is governed by a variety of gene products. This is consistent with studies of other mouse model systems, such as the Curly tail or Splotch mutants, which show variable penetrance and expressivity, depending on a combination of genetic and environmental factors[32]. The polygenic nature of neural tube closure is consistent with the fact that it is a complex morphological process involving changes in cell shape, migration and proliferation, and a subtle perturbation in any of these could produce the observed phenotype. It is also striking that the phenotype we observe is completely female-specific. Exencephaly more frequently affects female embryos than male embryos both in humans and model systems[17]. The reason for this sex-specificity remains elusive, although it may have a specific genetic basis that is related to the expression of genes which act in a completely female-specific manner.

It is possible that in wild-type embryos, *p53* functions only under rare circumstances of improper neural tube closure (8–16% of cases) to bring about some compensatory developmental mechanism. Alternatively, *p53* may be essential for the normal pattern of neural tube closure, and the survival of most *p53*-deficient embryos may reflect the existence of an alternative mechanism which is effective in only 80–90% of cases. Support for the latter possibility comes from studies of the SELH/Bc mouse strain, which also exhibits exencephaly at approximately 17% frequency[33]. All embryos of this strain fail to execute a particular aspect of neural tube closure, but in most cases an alternative pattern is adopted which is apparently equally effective[33]. Preliminary studies indicate, however, that there is no such obvious difference between the patterns of neural tube closure in wild type and *p53*-deficient embryos.

Precisely how lack of p53 function affects proper neural tube closure is unknown. Based on its known role in other contexts several models can be proposed. For example, *p53* has been shown to have critical roles both in mediating G1 arrest in response to DNA-damaging

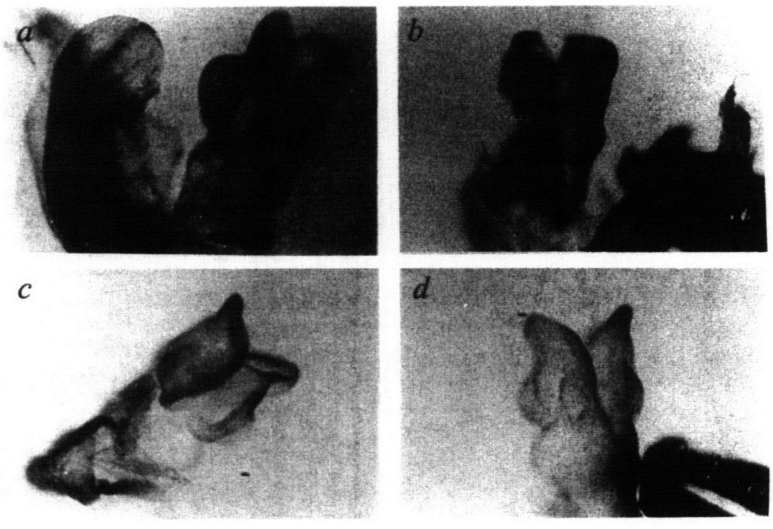
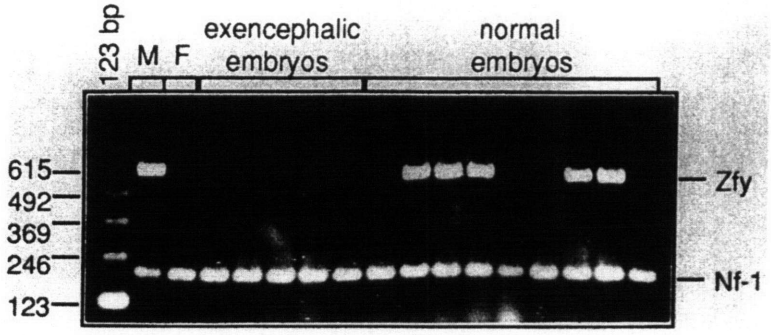


Fig. 4 Whole-mount TUNEL analysis of *p53*$^{-/-}$ embryos and littermate controls. *a*, Anterior view of 8–9 day *p53*$^{+/-}$ embryo stained to detect cell death. Note punctate nuclear staining along forebrain neural folds. *b*, Posterior view of 8–9 day *p53*$^{-/-}$ embryo stained to detect cell death. As in the *p53*$^{+/-}$ embryos, nuclear staining is along hindbrain neural folds. *c,d*, Negative control reactions: 8–9 day embryos stained in same way as other samples, but without TdT in the assay. Note the absence of specific staining.



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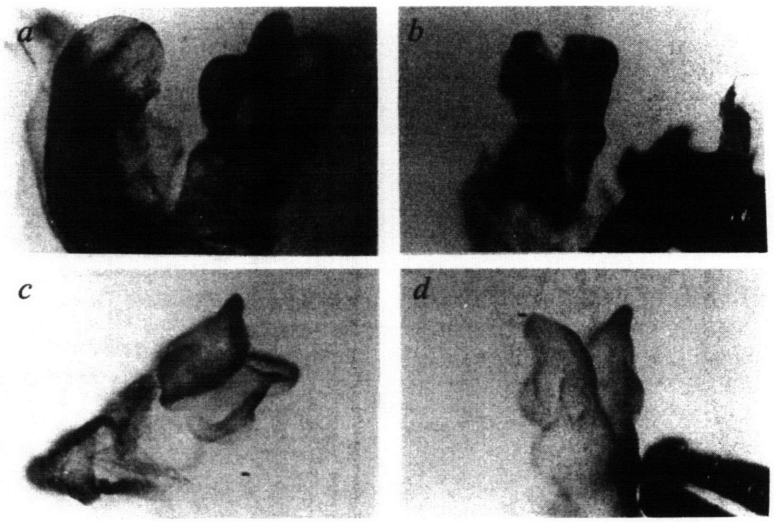

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agents[2,3] and in mediating apoptosis in response to DNA-damaging agents and/or oncoprotein expression[4–10]. In addition, *p53* can act as a sequence-specific transcriptional activator or a repressor[34] depending on the context. During neural tube closure, *p53* could potentially have a role in mediating cell cycle arrest either to limit cell proliferation or to prepare cells for a differentiation event important for closure. Indeed, incorrect regulation of cell cycling is thought to be the central cause of spina bifida in *ct* mutants[20]. Alternatively, *p53* deficiency could have a subtle effect on programmed cell death, which is not detectable in our assays. Finally, *p53* may simply regulate certain genes that are critical for neural tube closure. In fact, one known difference between wild-type and *p53* mutant embryos is the levels of the mRNA encoding the p21 cyclin-dependent kinase inhibitor[35]. p21 mRNA is elevated in the brain of *p53* mutant embryos, and this could potentially be significant in the induction of exencephaly.

The identification of *p53* as a novel player in the process of neural tube closure is also important because few factors involved in this process have been defined at the molecular level. Despite the fact that numerous mouse mutants have been characterized, few gene products have been identified. Only the Pax3 and Gli3 transcription factors have been identified as the affected genes in the Splotch (*Sp*) and Extra-toes (*xt*) mutants, respectively[36,37]. Characterizing the role of *p53* in neural tube closure should, therefore, augment our molecular understanding of neural tube closure. Determining the mechanism for the development of the defect will prove useful for designing therapy for neural tube disorders in humans. In addition, an elucidation of the function of *p53* in neural tube closure may lead to a better understanding of the role of this gene in tumour suppression.

Methods

Embryo analysis. $p53^{+/-}$ female mice were mated with either $p53^{+/-}$ or $p53^{-/-}$ males. Midday of the day on which the vaginal plug was detected was considered to be day 0.5 post-coitum (p.c.). Pregnant females were sacrificed from 12.5 to 18.5 days p.c. and embryos surgically removed for analysis. Embryos were fixed in 10% buffered formalin, dehydrated in graded solutions of ethanol, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

PCR analysis. Mouse embryos were harvested at between 12.5 and 18.5 days of gestation. Yolk sac DNA was obtained and used for *p53* genotyping[13] and sex determination for each embryo. Two sets of primers (one specific for the *Zfy* gene[23], another for the *Nf1* gene[22]) were used together in each of the PCR reactions for sex determination. DNA samples were taken through 30 cycles of amplification (30 s at 92 °C; 1 min at 55 °C; 1 min at 72 °C) in an MJ PTC-100 machine. Amplification products were visualized on a 2% agarose electrophoresis gel stained with ethidium bromide. The *Zfy* primers were forward 5'–AAGATAAGCTTACATAATCA-CATGGA–3', and reverse 5'–CCTATGAAATCCTTTGCTGCACA-TGT–3' (generously provided by David Page); the *Nf1* primers were: forward 5'–GGTATTGAATTGAAGCAC–3' and reverse 5'–TTCAATACCTGCCCAAGG–3' (ref. 22). For *p53* genotypes, DNA was obtained from tail preparations of 21-day-old mice and genotyped for *p53* status as described for the *p53*Δ mutation[13].

Whole mount TUNEL assay. The TUNEL assay was performed as described, with some modifications[38]: 8.0–9.0 day embryos were fixed in 10% buffered formalin and then transferred to 70% ethanol. Embryos were rehydrated and then treated for 1 h in a 4:1 solution of methanol:30% H_2O_2, followed by 3 washes with PBT (PBS with 0.1% Tween-20). Embryos were incubated 5 min in 15 μg ml^{-1} proteinase K and then two times 5 min in 2 mg ml^{-1} glycine. Embryos were fixed again in 4% paraformaldehyde, 0.2% glutaraldehyde in PBS for 1 hour and washed 3 times in PBT. The TdT reaction was done for 1 hour at 37 °C in a 50 μl reaction containing 1X TdT buffer, 10–15 U TdT (Gibco-BRL) and 20 μM digoxigenin-11-dUTP (Boehringer-Mannheim). The reactions were stopped by doing 4 washes in 300 mM NaCl, 30 mM Na Citrate, 0.1% Tween, over a 30 min period. The embryos were blocked in 10% fetal calf serum in PBT for 1 h. Anti-digoxigenin Fab fragment-HRP conjugate (Boehringer-Mannheim) was preabsorbed for 1 h with 1% fetal calf serum and 0.3% mouse or chick embryo powder in PBT. Embryos were incubated 1 h in preabsorbed antibody in PBT, at a concentration of 0.1–0.5 U antibody ml^{-1}. Embryos were washed 4 times in PBS and developed in HRP substrates (Peroxidase substrate kit, Vector Laboratories) until visible staining was detected. Embryos were then washed in PBS and photographed.

Acknowledgements

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APPENDIX B

Characterization of *Rb*-Deficient Fibroblast Growth

Notes of attribution

The data presented in this appendix represents work done by myself and Valerie (Tan) Sah, an undergraduate student whose research I supervised. Portions of this data were included in the following publication. A copy of this publication is included at the end of this appendix.

Herrera RH, Sah VP, Williams BO, Makela T, Weinberg RA, and Jacks T. (1996) Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in *Rb*-deficient fibroblasts. Mol Cell Biol. 16(5): 2402-2406

As discussed in Chapter 1, many of our insights into the function of pRB have come from studies performed on tumor derived cell lines. An inherent problem in such analysis is the fact that such tumor cell lines have undoubtedly acquired many mutations during both the initial process of tumor formation and the subsequent adaptation for growth *in vitro*. Thus, while tumor cell lines differing in the status of *Rb* (for example SAOS-2 and U2OS) have been utilized in side by side comparisons to assess *Rb* function (for example see (Gu et al., 1993)), the effect of other unknown genetic lesions on the specific function being studied is unclear. To help develop an *in vitro* system to overcome these problems, Valerie Tan, an undergraduate whose work I supervised, and myself characterized the growth of murine embryonic fibroblasts (MEFs) obtained from *Rb*-deficient embryos. Our work was included in a report of work done primarily by Ralph Herrera, a post-doctoral fellow in the laboratory of Robert Weinberg, which has been recently published in *Molecular and Cellular Biology* and is included at the end of this appendix.

Materials and Methods

Preparation of Murine Embryonic Fibroblasts

All cells used for the analysis described were derived from embryos purebred for the 129/Sv genetic background. *Rb* heterozygous females mated to *Rb* heterozygous males were sacrificed 12.5 days post-coitus and the embryos dissected from the uterus. Each embryo was separated and the yolk sac removed for eventual determination of *Rb* genotype via PCR (see Chapter 3). The head and internal organs were then dissected away from the remainder of the embryo. The remainder of the embryo was then rinsed in PBS and then placed in trypsin where it was minced with two sterile razor blades into a small aggregates of cells. These were then placed at 37 degrees for 30-45 minutes at which time 5 milliliters of fibroblast media (10% fetal calf serum, Pen/strep, in DME) were added. The resulting solution was then resuspended in a pipette 10 times and then added to 25 mls of fibroblast media in a T-75 flask. After growth to confluency, the cells were trypsinized from the T-75 flask by placing at 37 degrees for 10 minutes and then spun down. The

cell pellet was resuspended in fibroblast media and equal parts were added to two T-175 flasks containing 50 mls of fibroblast media. Upon growth to confluency in the T-175 flasks, the MEFs were trypsinized from the flask, spun down, and resuspended in freezing media. Each flask yielded approximately 20 vials of MEFs frozen at a concentration of approximately 2.5 X 10^6 cells.

Examination of growth kinetics of MEFs

This analysis was adapted from previously described protocols (Kastan et al., 1991). Early passage MEFs were grown at least one passage after thawing before experiments were performed. 1.5 X 10^6 cells were plated on a 10 cm plate and grown for 24-36 hours. The following procedure was then used to examine the cell cycle kinetics of the MEFs.

BROMODEOXYURIDINE / PROPIDIUM IODIDE ASSAY

1. Prepare BrdU solution: 20 ml DME mix + 20µl BrdU/Fdu (3 mg/ml BrdU and 0.3 mg/ml FdU); 0.45 µ filter into bottle.
2. Starting with 2 x 10^6 cells/plate, aspirate medium.
3. Pulse with 8 ml BrdU solution, and incubate for 4 hours at 37°C.
4. Aspirate off BrdU, and add 1ml trypsin/plate, incubating for 1minute at 37°C.
5. Quench reaction with 5 ml DME mix/plate; scrape with rubber policeman and transfer into 15 ml falcon tubes.
6. Count cells in hemacytometer.
7. Spin down in Sorvall RT6000 at 1400rpm at 4°C.
8. Aspirate, and wash 2 times with 3ml IFA/tube.
9. Fix cells with 70% MeOH at -20°C for 5 minutes. Add 2 ml MeOH/1 x 10^6 cells.
10. Spin for 10 minutes; aspirate.
11. Add 1 ml 0.1N HCl/0.7% Triton X 100 and incubate over ice for 10 minutes.
12. Add 2 ml PBSA; vortex and spin 10 minutes; aspirate and vortex pellet.
13. Add 0.5 ml dH_2O and then 16 µl 0.1N HCl. Heat at 97-100°C in water bath for 10 minutes.
14. Immediately transfer to ice bath on shaker for 10 minutes.
15. Add 1.5 ml IFA/0.5% Tween 20; spin, aspirate and vortex pellet.

16. Wash once with 2 ml IFA/0.5% Tween 20; spin and aspirate.
17. Add 100 µl BrdU-FITC (1:5 dilution in IFA) and incubate over ice and in the dark for 30 minutes.
18. Wash cells 1 time with 1 ml IFA/0.5% Tween 20; spin and aspirate.
19. Add 1 ml IFA/0.5% Tween 20 + 1µl RNase A, and incubate in 37°C water bath for 15 minutes.
20. Add 10 µl 50 mg/ml propidium iodide and incubate on ice and in the dark overnight.
21. After the incubation, run the cells on a fluorescence activated cell sorter (FACS).

Concomitant with the BrdU/PI analysis, doubling times were calculated for these cell lines. 3.0×10^5 cells were plated onto several different 6 well wells. Then, every 24 hours, two wells was trypsinized and the total cell number recorded. Doubling times calculated in this manner were used with the data from the BrdU/PI FACS analysis to calculate the number of hours the average cell in the population of MEFs spent in each phase of the cell cycle. In addition, cells were analyzed for physical size by plotting forward versus side scatter on the FACS machine.

Results

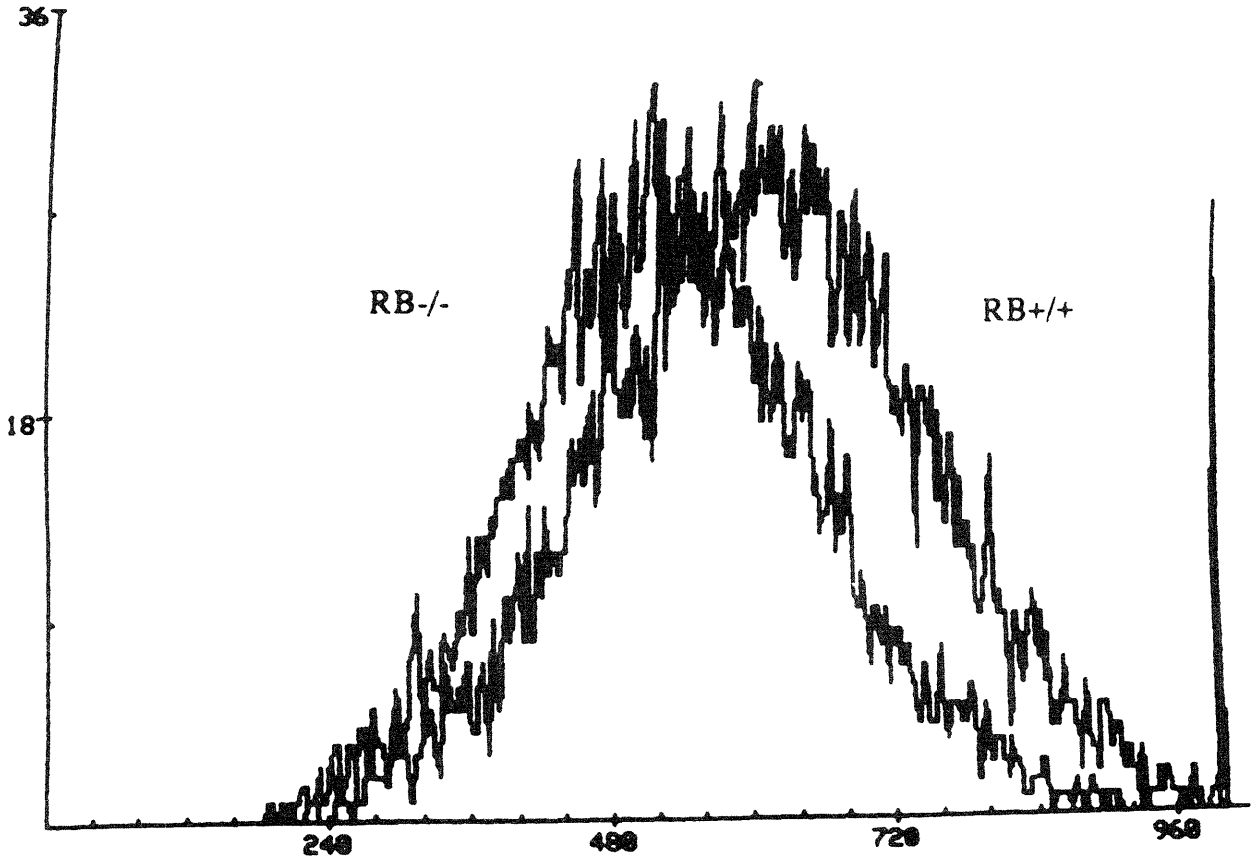
Cell Size Determination

The FACS analysis of these MEF populations revealed that Rb-deficient cells had a diameter approximately 85% of their wild-type counterparts and this calculated to a reduction in volume of about 40%. A representative FACS analysis of this determination is shown in Figure B-1 on the following page.

Figure B-1

Relative Size of Rb +/+ and Rb -/- MEFs.

The graph below shows an example of forward versus side scatter FACs analysis of Rb +/+ and Rb -/- MEFs.



Cell Cycle Kinetics

The results of the experiments on cell cycle kinetics are summarized in the tables below.

e B-1

mary of Cell cycle kinetics experiments

are shown +/- standard deviation. For Rb +/+ cells data from 14 independent experiments representing 6 independent ce
. For Rb -/- cells data from 16 independent experiments representing 7 independent cell lines was used.

F otype	Doubling Time (Hours)	% G0/G1	G0/G1 Time (hours)	% S	S Time (Hours)	% G2/M	G2/M Time (Hours)
+/+	26.60 +/- 9.1	65.5 +/- 6.9	17.4 +/- 6.3	18.7 +/- 7.1	4.9 +/- 2.2	15.6 +/- 4.9	4.2 +/- 2.2
-/-	26.54 +/- 7.8	45.7 +/- 7.8	12.1 +/- 2.8	31.2 +/- 7.8	8.2 +/- 2.0	22.2 +/- 9.4	6.0 +/- 3.2

Discussion

The data presented in this appendix suggest that although the tissue from which *Rb*-deficient MEFs are derived (embryonic body wall) appears normal, the MEFs themselves exhibit intrinsic differences from wild-type MEFs. Examination of normal cell cycle kinetics from exponentially growing cell populations revealed a shortening of G1 phase in the *Rb*-deficient cells with a concomitant compensatory lengthening of S phase. This difference is also associated with a decrease in the cell size. Perhaps some defect associated with a disruption in restriction point modulation causes the cells to prematurely enter S phase before the necessary metabolites have been built up to ensure a normal progression through S phase. The cell may compensate for this by increasing the length of S phase to allow the necessary metabolite levels to build up.

The decreased cell size probably also is due to the premature entry into S phase. Other examples of alterations which cause a decrease in cell size include fibroblasts in which *cyclin E* is ectopically expressed (Ohtsubo and Roberts, 1993). This similarity between the cyclin E-overexpressing cells and the *Rb*-deficient cells was examined further by Ralph Herrera, who showed that the general regulation of cyclin E levels was significantly altered in *Rb*-deficient MEFs.

In perhaps the most important observation associated with the analysis of the MEFs, Ralph Herrera showed that some aspects of restriction point regulation in *Rb*-deficient MEFs were altered. While the restriction point as defined by sensitivity to serum withdrawal is not significantly altered, *Rb*-deficient MEFs are not sensitive to low levels of cycloheximide as are their wild-type counterparts. The differences of *Rb*-deficient MEFs in responding to these two assays to measure the restriction point are not clear at this time.

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Altered Cell Cycle Kinetics, Gene Expression, and G_1 Restriction Point Regulation in *Rb*-Deficient Fibroblasts

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Fibroblasts prepared from retinoblastoma (*Rb*) gene-negative mouse embryos exhibit a shorter G_1 phase of the growth cycle and smaller size than wild-type cells. In addition, the mutant cells are no longer inhibited by low levels of cycloheximide at any point in G_1 but do remain sensitive to serum withdrawal until late in G_1. Certain cell cycle-regulated genes showed no temporal or quantitative differences in expression. In contrast, cyclin E expression in *Rb*-deficient cells is deregulated in two ways. Cyclin E mRNA is generally derepressed in mutant cells and reaches peak levels about 6 h earlier in G_1 than in wild-type cells. Moreover, cyclin E protein levels are higher in the $Rb^{-/-}$ cells than would be predicted from the levels of its mRNA. Thus, the selective growth advantage conferred by *Rb* gene deletion during tumorigenesis may be explained in part by changes in the regulation of cyclin E. In addition, the mechanisms defining the restriction point of late G_1 may consist of at least two molecular events, one cycloheximide sensitive and pRb dependent and the other serum sensitive and pRb independent.

The retinoblastoma (*Rb*) tumor suppressor gene is inactivated in a wide range of human tumors. Its encoded protein pRb, has been implicated in cell cycle regulation and is known to regulate members of the E2F family of transcription factors. These factors, in turn, control the expression of a variety of genes expressed in the G_1 and S phases of the cell cycle (34). Therefore, loss of *Rb* function might be expected to result in deregulated gene expression and abnormal cell cycle progression.

pRb has been shown to be phosphorylated at a particular stage of the cell cycle (2, 4, 6, 11). This phosphorylation occurs late in G_1 and is responsible for the inactivation of pRb. This event is thought to be critical for passage into S phase. Therefore, a characterization of the mediators of pRb phosphorylation and of the consequences of this event is central to an understanding of the molecular details of cellular progression through G_1 and into S.

Recently it has been shown that cyclin-dependent kinases (CDKs), in concert with their regulatory cyclin subunits, can phosphorylate pRb (10, 14, 16). The cyclin CDKs of particular interest are cyclin E·CDK2 and cyclin D·CDK4,6. These molecules are active in G_1, the time of pRb phosphorylation, and indeed have been shown to phosphorylate pRb in vitro and in transfection studies. In addition, these regulatory proteins are essential for transit through G_1, and their premature activation leads to an accelerated G_1 (28, 31). Thus, cyclin CDKs are prime candidates for the pRb kinases.

Also occurring late in G_1 is the mammalian restriction (R) point (29). The R point was defined by Pardee (29) as the position in G_1 beyond which cells are committed to continue into S phase. Three criteria are used to define this point. After the R point, cells (i) no longer need serum to continue into S, (ii) are no longer sensitive to inhibition by transforming growth factor β and (iii) are no longer sensitive to low levels of the protein synthesis inhibitor cycloheximide. Therefore, beyond the R point, cells are no longer responsive to inhibitory signals and no longer need stimulatory signals for progression into S.

The precise roles of pRb in controlling cell proliferation have been difficult to define, since experiments designed to analyze pRb function usually have been conducted with tumor cells that have suffered a number of other mutations in addition to alteration of *Rb*. These other changes complicate analysis of the role of pRb itself. To circumvent this problem, we have analyzed fibroblasts prepared from *Rb*-deficient mouse embryos; these cells are genetically matched with those prepared from wild-type embryos with the exception of the known mutation in *Rb* itself (15).

MATERIALS AND METHODS

Cell cycle analyses. Early-passage (<5) primary fibroblasts isolated from *Rb* mutant and wild-type embryos at 12.5 days of gestation (15) were used in all experiments. Experiments were carried out by comparing cells at identical passage numbers obtained from littermate embryos. Cells were labeled with bromodeoxyuridine-propidium iodide and analyzed on a Becton Dickinson FACScan for cell cycle or size analysis. For [^{3}H]thymidine incorporation experiments, cells were starved in serum-free Dulbecco modified Eagle medium for ~48 h before being restimulated with 15% serum for the indicated times. [^{3}H]thymidine labeling was for 30 min before lysis and counting.

Immunoblot analyses and kinase assays. Immunoblot analyses were performed by standard procedures (1). Antibodies to cyclin proteins were from rabbit polyclonal serum. Specificity of the antibodies was confirmed by detection of recombinant cyclin proteins and migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Kinase assays were performed as described previously (22), using equal amounts of protein for each immunoprecipitation. Antibodies to CDK2 were from Santa Cruz Biotechnology. Quantification of the kinase assay was by densitometric scanning.

RNA analyses. RNA purification and Northern (RNA) analyses were performed by standard procedures (1). The probes were derived from cDNAs of the respective genes. Quantification was by densitometric scanning.

RESULTS AND DISCUSSION

Microscopic examination and flow cytometric analysis revealed that the diameter of the *Rb* cells was approximately 85% of that of the wild-type cells (Fig. 1A). The calculated 40% smaller volume of the *Rb* cells suggested a shorter

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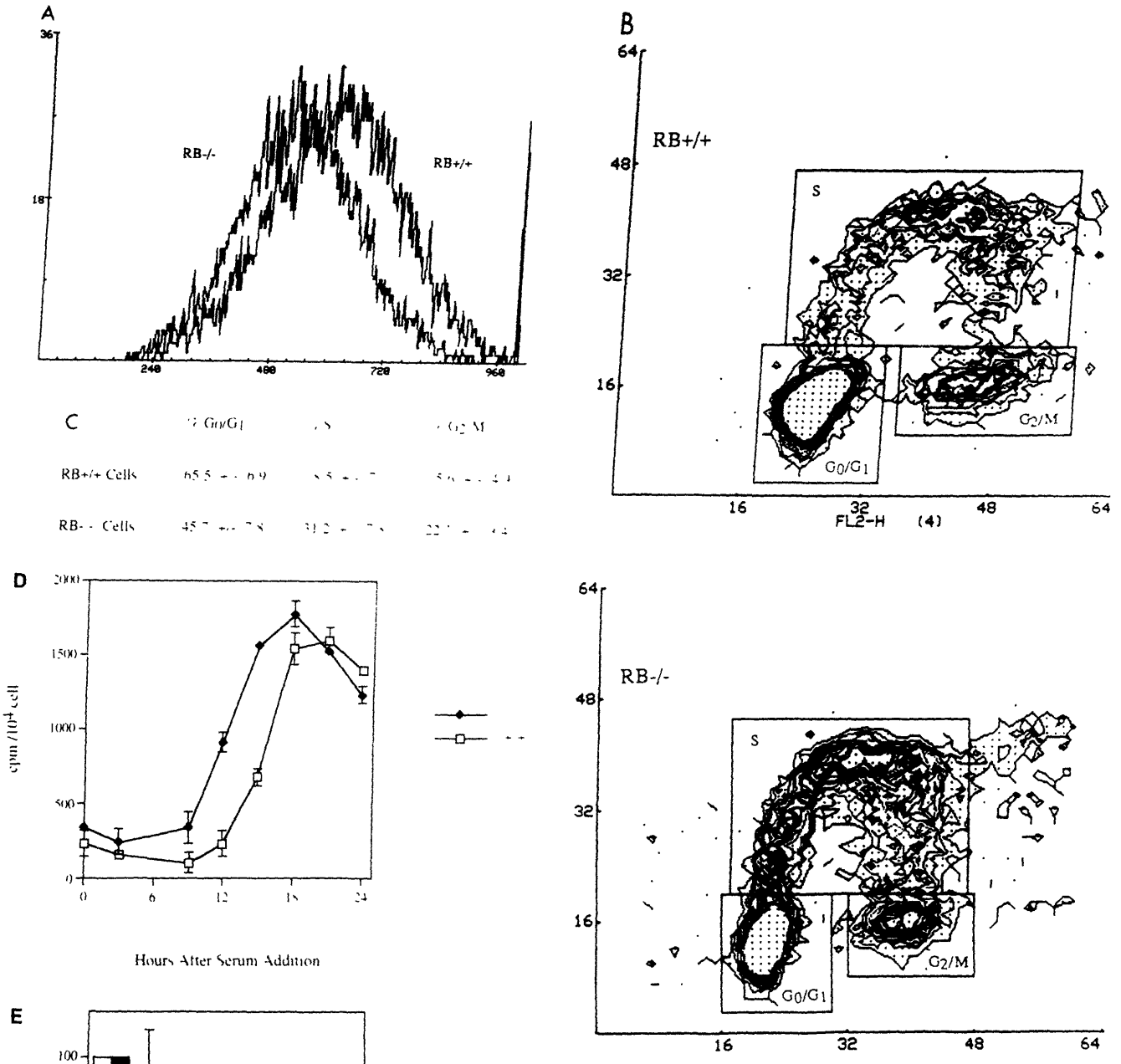

FIG. 1. Altered size and cell cycle kinetics in *Rb* fibroblasts. (A) Forward versus side-scatter flow cytometric analysis. Size is plotted on the x axis, and cell number is plotted on the y axis. Curves from mutant and wild-type cells are indicated. (B) FACScan analysis of asynchronously growing mutant and wild-type cells labeled with bromodeoxyuridine-propidium iodide to determine the percentage of cells in each cell cycle phase. The G_0, G_1, G_2 M, and S phases are labeled. (C) Summary of FACScan analyses, with standard deviations from a total of 14 and 16 experiments from *Rb*$^{+/+}$ and *Rb*$^{-/-}$ cells, respectively. The observed compensatory increase in S seen in the mutant cells has also been found in fibroblasts overexpressing cyclin E (28). (D) [^{3}H]thymidine incorporation of mutant and wild-type cells labeled at different times after release into G_1 by readdition of serum to starved cells. Data are derived from an experiment performed in triplicate; standard deviations are indicated by error bars. Similar results were obtained from cells derived from at least 10 different *Rb*$^{+/+}$ and *Rb*$^{-/-}$ embryos. (E) Thymidine incorporation of mutant and wild-type cells labeled after growth in reduced concentrations of serum. Cells were grown for 3 days at the serum concentrations indicated and then labeled with [^{3}H]thymidine. The y axis is expressed as the percent incorporation of cells grown in 15% serum. Data shown were derived from an experiment performed in triplicate; standard deviations are indicated by error bars.

growth cycle. Therefore, we determined the length of the cell cycle phases by flow cytometry of asynchronously growing cells. As shown in Fig. 1B and C, while the percentages of *Rb* and wild-type cells in G_2 were comparable, there was a 20% reduction in the number of mutant cells in G_1 and a concomitant increase of 12% of those in S. Since the percentage of cells in each cell cycle phase reflects the time required for each cell type to transit that phase, we concluded that pRb influences the timing of G_1 and that its absence leads to an abbreviated G_1 and reduced cell size. However, because of a lengthened S phase, the overall doubling time of the pRb-deficient cells was comparable to that of their wild-type counterparts (not shown).

To confirm these altered cell cycle kinetics, mutant and wild-type cells were starved of serum and then released from G_0 by the readdition of serum. S-phase entry was monitored by measuring incorporation of [^{3}H]thymidine into DNA. Mutant cells synchronized in this fashion exhibited a G_1 phase that is 3 to 4 h shorter than that of their wild-type counterparts (Fig. 1D), reinforcing the results from flow cytometry. Together, these observations provide direct evidence indicating that pRb indeed is an important regulator of cell cycle progression.

It was of interest to determine if the mitogenic effects of serum worked in part through inactivation of pRb. Therefore, we determined if the *Rb* cells had obtained some level of serum independence for growth. *Rb* and *Rb* cells were grown in various concentrations of serum for 3 days before being pulse-labeled with [^{3}H]thymidine. As shown in Fig. 1E, the two cell types exhibit very similar degrees of labeling at all concentrations of serum used. This result suggests that while serum stimulation of growth may work in part by inactivation of pRb, other molecular events are required for cells to proceed through the cell cycle.

As described above, the critical checkpoint of G_1 has been termed the R point by Pardee (29). The R-point transition and the onset of pRb phosphorylation and inactivation occur at similar times in mid/late G_1, suggesting a connection between these two processes. Therefore, it was of interest to determine whether R-point control was maintained in the *Rb* cells. Mutant and wild-type cells were growth arrested by serum starvation and then allowed to reenter the cell cycle by readdition of serum. At various points thereafter, either serum was removed (Fig. 2A) or cycloheximide was added to the cells to a final concentration of 50 ng ml (Fig. 2B). The R point, as defined by escape from serum dependence, was found to occur at similar times relative to the onset of S phase in both cell types (Fig. 2A). Because the *Rb* cells progress through G_1 more rapidly than wild-type cells and enter S phase 3 to 4 h earlier following serum addition (Fig. 1D), the R point is similarly shifted by this amount of time relative to controls (Fig. 2A). Importantly, the *Rb* cells remain dependent on serum factors up to the R-point transition (Fig. 1E). In striking contrast, the cycloheximide R point appeared to be largely if not completely absent in the *Rb* cells (Fig. 2B); thus, *Rb* cells escaped cycloheximide inhibition rapidly after serum restimulation. These results indicated that the R-point transition depends on at least two distinct, separable molecular processes, one cycloheximide sensitive and pRb dependent and the other serum sensitive and pRb independent.

A shorter G_1 and smaller size have also been observed in cells that overexpress several of the G_1 cyclin genes (28, 31–33). For this reason, we determined the levels of cyclin proteins at various points of the cell cycle in the *Rb* and wild-type cells by immunoblotting. The expression of G_1 cyclins was variably affected by the absence of pRb (Fig. 3A). Cyclin D1 levels were slightly elevated (approximately two- to fourfold) in

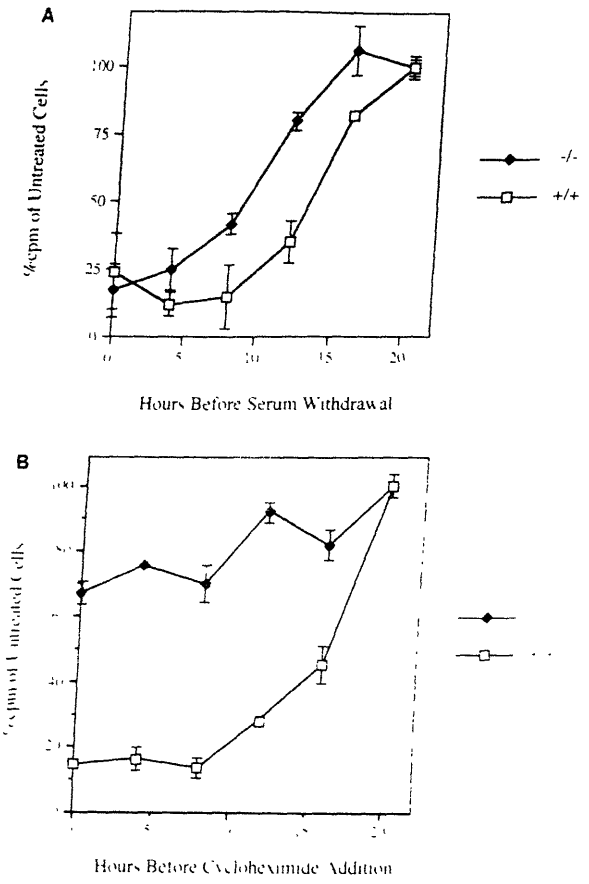

FIG. 2. Analysis of the R point of late G_1 in *Rb* and *Rb* fibroblasts. (A) Mutant and wild-type cells were growth arrested by serum starvation and then allowed to reenter the cell cycle by readdition of serum. At various points thereafter, serum was removed. Entry into S was measured by [^{3}H]thymidine incorporation at 20 h after serum restimulation. (B) As described for panel A except that instead of serum removal, cycloheximide was added to the cells to a final concentration of 50 ng ml. The *y* axis is expressed as percent counts per minute of samples serum stimulated for 20 h without subsequent serum withdrawal or cycloheximide addition. Results from representative experiments are shown. Data shown were derived from an experiment performed in triplicate; standard deviations are indicated by error bars. Similar results were obtained from cells derived from at least 10 independent *Rb* and *Rb* embryos.

the *Rb* cells at different points in G_1. In contrast, cyclin E protein levels were vastly increased in the pRb-deficient cells regardless of their position in G_0 or G_1. Quantitation of autoradiograms indicated that the mutant cells contain approximately 10 times more cyclin E protein (data not shown). In addition, the slight induction of cyclin E protein levels occurring in wild-type cells late in G_1 was observed earlier in the *Rb* cells (Fig. 3A; compare 18- and 24-h time points in wild-type cells with 6- and 12-h time points in mutant cells). Cyclin A protein levels were slightly elevated in the mutants early in G_1 as well as in G_0. These observed increases could be a consequence of the premature and highly elevated expression of cyclin E in the pRb-deficient fibroblasts or a direct regulation of cyclin A expression by pRb.

We next addressed whether the increased levels of the cyclin E and A proteins present in the mutant cells also led to a corresponding increase in CDK2 kinase activity. Accordingly, CDK2 complexes were immunoprecipitated from wild-type and pRb-deficient cells and used to phosphorylate histone H1

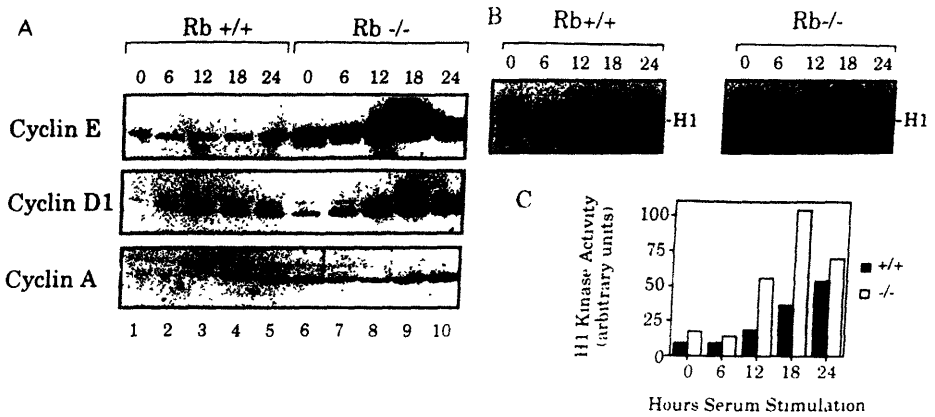

FIG. 3. (A) Immunoblot analysis of protein lysates prepared after the readdition of serum to starved cells. Blots contain total protein lysates from cells stimulated with serum for the indicated times. Cyclin proteins were detected by polyclonal rabbit antibodies. Protein amounts were normalized by spectrophotometric methods and Ponceau S staining of the nitrocellulose filters. (B) Histone H1 kinase activity of anti-CDK2 immunoprecipitates prepared after the readdition of serum to starved cells for the indicated times. (C) Quantification of the anti-CDK2 immunoprecipitable histone H1 kinase activity.

in vitro. Figures 3B and C show that immunoprecipitates from the *Rb* cells display higher H1 kinase activities throughout G_1. In addition, the peak of kinase activity occurs earlier in the mutant cells, and this difference is comparable to their shortening of G_1. It should be noted that the kinase activities were measured by using CDK2 antibodies in the immunoprecipitations. Therefore, both cyclin A and cyclin E could have contributed to increased CDK2 activity. In addition, as in wild-type cells, *Rb* cells undergo a serum-dependent activation of CDK2 function, despite constitutive high levels of cyclin E protein. Indeed, this pRb-independent kinase activation may account for the observed serum dependence of the *Rb* cells (Fig. 1E and 2A).

We next performed Northern blot analysis of RNA prepared at various times after the readdition of serum to determine if the dramatic increase in cyclin E protein levels in the *Rb* cells was due to differences in levels of cyclin E mRNA. Expression of the cyclin E gene has been shown to be normally induced in late G_1 several hours before entrance into S phase (8, 11, 20, 21). As shown in Fig. 4A and C, peak levels of cyclin E mRNA expression were seen 6 h earlier in the mutant cells. This difference is comparable to the overall shortening of G_1 seen in the *Rb* cells. In addition, cyclin E mRNA levels were elevated in all parts of G_1 in the mutant cells. However, as in the CDK2 kinase activity profile (Fig. 3), an induction of cyclin E mRNA occurs in the *Rb* cells, suggesting that the cyclin E gene is under complex transcriptional control, only a part of which is responsive to pRb inactivation.

Work of others has demonstrated that the ectopic expression of cyclin E causes a substantially shortened G_1 phase of the cell cycle, smaller cells, and a longer S phase (28, 32, 33). These observations suggest that the entrance into the late phase of G_1 is controlled by the availability of cyclin E mRNA and protein and can be advanced by prematurely elevated protein levels. The pRb-deficient cells show a phenotype strikingly similar to that of these cyclin E-overproducing cells, including elevated cyclin E protein levels at all points of the cell cycle. It is interesting that elevated levels of cyclin E have recently been observed in several human tumors (17, 18).

Transcriptional up-regulation of the *TK* and cyclin A genes, which normally follows cyclin E induction, was also accelerated in the *Rb* cells (Fig. 4A and data not shown). Whether this is a direct result of the absence of *Rb* function or an indirect effect of the overexpression of cyclin E is unclear.

In contrast to the changes in gene expression observed in mid late G_1, transcription of the immediate-early gene *c-fos* and transcription of two delayed-early genes, *c-myc* and the cyclin D1 gene, showed identical patterns (Fig. 4A and B). This finding suggests that pRb does not exert any direct control on gene expression early in G_1 and thus contrasts with results of others implying pRb-mediated control of *c-fos* and the cyclin D1 gene and E2F-mediated control of *c-myc* (13, 19, 24, 35, 36). Therefore, the influence of pRb on the timetable of G progression appears to be limited largely to its effects on the timing of the induction of cyclin E mRNA and subsequently occurring processes. This suggests, in turn, that the G_1 phase of the cell cycle is divided into two periods demarcated by the mid late G_1 event of cyclin E mRNA induction.

As described above, the peak levels of cyclin E mRNA are about twofold higher in the *Rb*-deficient cells but the levels of protein are about 10 times higher. This disparity suggests that pRb may also influence posttranscriptional control mechanisms governing cyclin E expression. The nature of these mechanisms and their regulation by pRb are unknown at this time.

The detailed mechanisms underlying the negative regulation of cyclin E expression by pRb are not revealed by the present work. One possibility is suggested by the observation (10a) that the cyclin E gene promoter contains a number of E2F sites, the activity of which may be inhibited by pRb. Independent of mechanism, the observed ability of pRb to antagonize cyclin E expression would seem to stand in opposition to earlier studies demonstrating that cyclin E operates to antagonize pRb function (14). In this earlier work, ectopic expression of cyclin E was found to cause the hyperphosphorylation of pRb and its functional inactivation. The two sets of observations can be reconciled by the existence of a self-reinforcing, positive feedback loop that is established in late G_1, in which cyclin E participates in the functional inactivation of pRb while the resulting inactivated pRb permits the ongoing expression of cyclin E. We note that a positive feedback loop has also been proposed to describe the control of late G_1 transit in the yeast *Saccharomyces cerevisiae* and fruit flies (5, 7, 9, 25–27).

Unexplained by the present work is the disparity between the reduced G_1 phase of the pRb-deficient fibroblasts in culture and the relatively normal development of *Rb* embryos up until day 13 of gestation (15), particularly in light of the presumed importance of G_1 in various growth control and

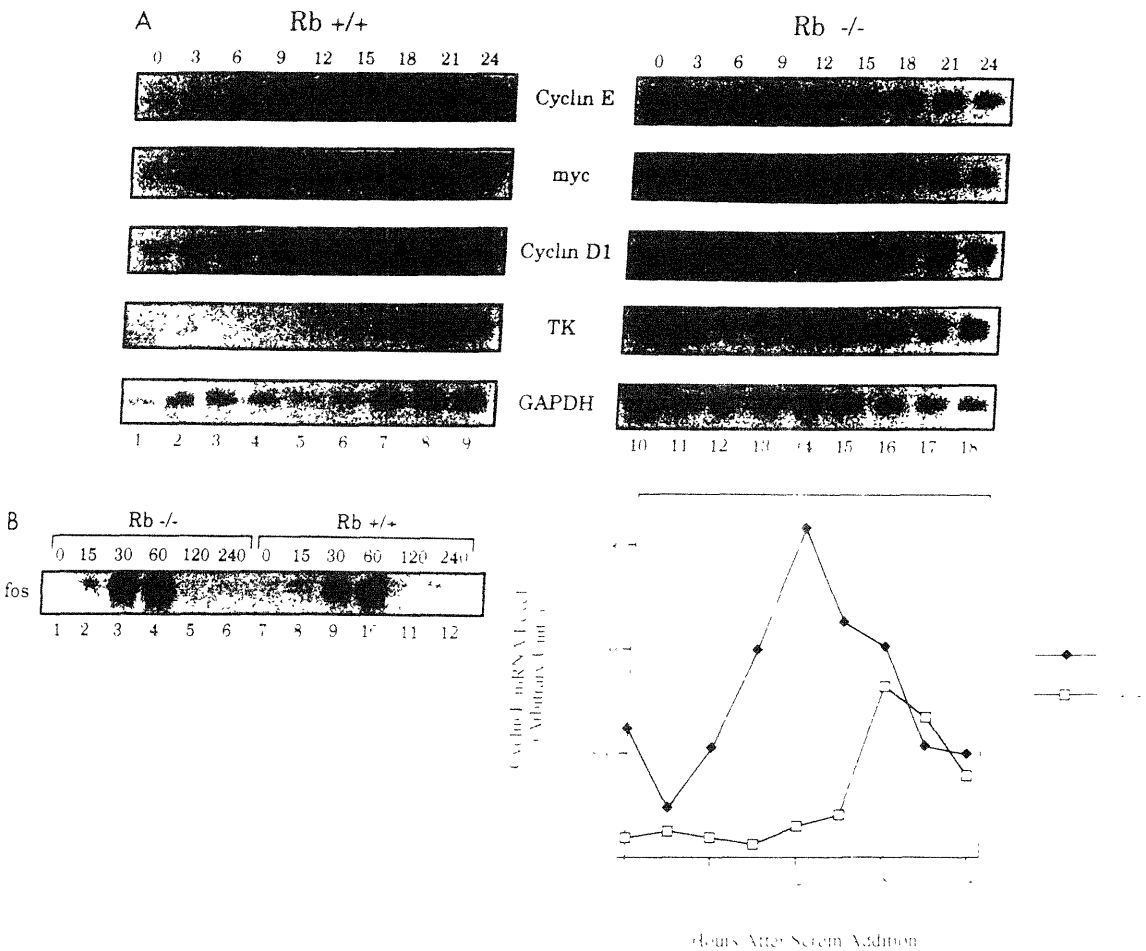

FIG. 4. Northern blot analyses of RNA prepared from [illegible] with serum for the indicated times (hours) were hybridized to the radiolabeled cDNA probes. Sources of RNA [illegible] As shown by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [illegible] RNA was loaded in the wild-type [illegible] 24-h samples. (B) A blot containing total RNA purified from cells stimulated with serum [illegible] to the third exon of the human gene. Equal loading was ensured [illegible] Northern blot in panel A.

differentiation mechanisms. In addition, the relatively normal transcriptional induction of the cell cycle-regulated genes *c-fos*, *c-myc*, and the cyclin D gene observed in pRb-deficient fibroblasts suggests that these genes may be regulated by other non-pRb proteins.

Evidence from a variety of sources indicates that the R point represents a cell cycle landmark that is of central importance in cell growth control (29). Indeed, it has been shown that transformed cells are more resistant to cycloheximide than are their nontransformed counterparts (3, 23, 30). Yet other results indicate that the pRb control mechanisms are disrupted by a variety of ways in many and possibly all types of tumor cells (37). By at least one operational definition—cycloheximide inhibition—R-point control appears to be absent in *Rb* mouse embryo fibroblasts. This observation, together with the observed contemporaneity of pRb phosphorylation and the R-point transition, suggests that pRb may mediate control of this important growth-regulating checkpoint.

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APPENDIX C

Phenotypes of Mice Carrying Targeted Mutations

in Both *Rb* and *p107*

The work described in this appendix has been accepted for publication in Genes and Development.

Notes of attribution

The *p107* gene disruption and the analysis described was performed by Myung-Ho Lee, a post-doctoral fellow in the laboratories of Ed Harlow and Nick Dyson, in collaboration with Tyler Jacks. The analysis of the phenotypes in mice carrying mutations in both genes were performed jointly by myself and Myung-Ho Lee, with assistance from George Mulligan.

As briefly described in Chapter 1, *Rb* is a one of a three member gene family which also includes *p107* and *p130* (reviewed in Cobrinik, 1996). To assess whether *p107* and *p130* play essential roles in murine development or can act as tumor suppressor genes in some capacity, several investigators working in collaboration created mice with targeted inactivated mutations in both genes. *p130*-deficient mice were created by David Cobrinik, Greg Hannon, David Beach, Robert Weinberg, and Tyler Jacks and found to have no detectable phenotype. *p107*-deficient mice were created by Myung-Ho Lee, Nick Dyson, Ed Harlow, and Tyler Jacks and were similarly found to have no detectable phenotype.

To examine whether *Rb*, *p107*, and *p130* had overlapping functions in murine development, several people began to cross the strains and analyze all the combinations of animals deficient for two of three genes. My contribution to this was to collaborate with Myung-Ho Lee to analyze the phenotypes associated with mice deficient for both the *p107* and *Rb* genes. Our analysis indicated mice carrying mutations in both *Rb* and *p107* did indeed show synergistic defects. *Rb+/-;p107-/-* mice are severely runted and most die neonatally. Those that do survive exhibit severe multifocal retinal dysplasia and surviving females exhibit vaginal atresia. *Rb-/-;p107-/-* embryos fail a full 1-2 days earlier than *Rb-/-* embryos and display earlier defects in liver and nervous system development. The report of this analysis follows.

Targeted Disruption of *p107*: Functional Overlap Between *p107* and *Rb*

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Abstract

To explore the physiological role of *p107*, a member of retinoblastoma gene (*Rb*) family, we disrupted the mouse gene by homologous recombination in embryonic stem cells. *p107* homozygous mutant mice were viable, fertile, and displayed no obvious abnormalities. To investigate possible functional overlap between *p107* and *Rb*, mice with mutations at both loci were generated. $Rb^{+/-};p107^{-/-}$ mice have a pronounced growth retardation and increased mortality rate during the first 3 weeks after birth. The $Rb^{+/-};p107^{-/-}$ pups that survive to adulthood did not show any altered tumor predisposition when compared to $Rb^{+/-}$ mice but developed multiple dysplastic lesions of the retina. Embryos homozygous for both *Rb* and *p107* died at approximately 11.5 days of gestation, two days earlier than embryos homozygous for *Rb* alone. Histological examination revealed accelerated apoptosis in the liver and the central nervous system of $Rb^{-/-};p107^{-/-}$ embryos relative to $Rb^{-/-}$ embryos. These results provide the first *in vivo* evidence that *p107* and *Rb* have overlapping functions in some tissues of the developing and adult mouse, particularly with respect to apoptosis but not tumorigenesis.

Introduction

The retinoblastoma gene (*RB*) is a prototype of the tumor suppressor genes. In humans, mutation of the *RB* is frequently found in tumors, and the inactivation of both copies of the gene appears to be the rate-limiting event in the genesis of retinoblastoma (Cavenee et al., 1983; Dryja et al., 1984; Friend et al., 1986; Lee et al., 1987). In addition, mutation of genes that function in the regulation of the retinoblastoma protein (pRB) also occurs frequently in human tumors, suggesting that the deregulation of the normal pathway in which pRB functions is a common and important event in many types of tumorigenesis (Friend et al., 1986; Fung et al., 1987; Harbour et al., 1988; Lee et al., 1988; T'Ang et al., 1988; Yokota et al., 1988; Horowitz et al., 1989 and 1990; Toguchida et al., 1989). Inactivation of *Rb* by gene targeting in the mouse has revealed that pRB is required for normal development. Embryos homozygous for mutant *Rb* die between 13.5 and 15.5 days of gestation with extensive apoptotic cell death in the liver, central nervous system, and ocular lens as well as inefficient definitive erythropoiesis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Morgenbesser et al., 1994). Adult mice heterozygous for the *Rb* mutation do not develop retinoblastoma but are highly predisposed to intermediate lobe pituitary tumors and medullary carcinoma of the thyroid (Jacks et al., 1992). Analysis of chimeric $Rb^{-/-}$ mouse embryos suggests that $Rb^{-/-}$ retinoblasts may undergo cell death instead of proliferating (Maandag et al., 1994). However, retinoblastoma has been induced in the mouse by the targeted expression of SV40 large T (Windle et al., 1990; Al-Ubaidi et al., 1992), or human papillomavirus type 16 (HPV-16) E7 and E6 (Howes et al., 1994), both of which inactivate *Rb* family proteins and p53. This raises the possibility that mutations in other members of the *Rb* family and *p53* are further required for induction of retinoblastoma in the mouse.

The *p107* and *p130* genes encode proteins with related structures and similar biochemical properties to pRB. Much of the sequence similarity among pRB, p107, and p130 resides in the domains that mediate interaction with viral oncoproteins. The transforming regions of adenovirus E1A, SV40 large T antigen and HPV-16 E7 proteins target these *Rb* family proteins. The *Rb*

gene family is expressed in most cell types, and like pRB, the overexpression of either p107 or p130 has been shown to arrest cells in G_1 phase of the cell cycle (Zhu et al, 1993; Claudio et al., 1994). All three proteins have been shown to associate with the transcription factor E2F *in vivo* and to repress E2F-dependent transcription when overexpressed. The co-expression of *E2F* genes reverses the cell cycle arrest caused by pRB (Zhu et al., 1993; Qin et al., 1995), p107 (Zhu et al., 1995) or p130 (Vairo et al., 1995). Thus, like pRB, p107 and p130 may also be negative regulators of cell proliferation, acting at least in part through regulation of the E2F transcription factor.

Despite the extensive similarities among pRB, p107, and p130, there are several indications that these proteins have distinct properties. pRB, p107, and p130 appear to bind to different subsets of E2F and at different phases of the cell cycle. p107 interacts preferentially with E2F-4 in cycling cells (Ginsberg et al., 1994; Beijersbergen et al., 1994a), whereas pRB interacts with E2F-1, E2F-2, and E2F-3 (Lees et al., 1993) and p130 with E2F-4 (Vairo et al., 1995) and E2F-5 (Hijmans, 1995). Cyclin A/cdk2 or cyclin E/cdk2 are found in most p107/E2F and p130/E2F complexes but not in pRB/E2F complexes (Cao et al., 1992; Ewen et al., 1992). Furthermore, p130/E2F complexes are detected in cells in G_0, whereas the pRB/E2F and p107/E2F complexes appear later in G_1 and persist in S phase cells (Shirodkar et al., 1992; Cobrinik et al., 1993; Schwarz et al., 1993). Possible functional differences between *p107* and *Rb* are further supported by *in vitro* data showing that ectopic expression of *p107* suppresses proliferation of the human cervical carcinoma cell line C33A, whereas overexpression of *Rb* does not (Zhu et al., 1993). A role for *p107* in embryonic development is also suggested by its distinctive expression pattern in the heart, which differs from that of *Rb*. The *p107* gene is expressed at high levels in the heart during embryonic development, and transcript levels then decrease significantly at birth, whereas the *Rb* gene is expressed steadily at low levels during the same period (Kim et al, 1994).

Despite considerable *in vitro* evidence for the involvement of p107 in growth control and development, there is no proof supporting such a function *in vivo*. Importantly, it is uncertain

whether *p107* can act as tumor suppressor gene, because no specific mutation of *p107* has been reported in tumors to date. To address whether p107 is involved in growth control and development *in vivo* and to investigate the functional relationship between p107 and pRB, we have created *p107* mutant mice by gene targeting and generated *Rb* and *p107* double mutant mice. We have determined that while deficiency for p107 does not induce obvious abnormalities in mice or the cell cycle profile in cultured cells, it alters the phenotype of the *Rb* mutation, particularly with respect to apoptosis and retinal pathogenesis.

Results

p107 gene targeting

The *p107* gene was inactivated in murine embryonic stem (ES) cells by homologous recombination as shown in Fig. 1. A targeting vector (Fig. 1A) was constructed in which a fragment containing a 95 bp 3' region of exon 1 and a 2.3 kb region of the downstream intron was replaced by a PGK-*neo* expression cassette, containing the neomycin resistance gene driven by the phosphoglycerate kinase promoter, in the anti-sense orientation relative to the coding region of *p107*. A herpes simplex virus thymidine kinase gene expression cassette (PGK-*tk*) was also placed adjacent to the 5' end of the homology to allow the use of both positive and negative selection (Mansour et al., 1988). Homologous recombination was predicted to produce a mutant allele capable of synthesizing only the first 22 amino acids of the wild-type p107 sequence.

D3 ES cells (Gossler et al., 1986) were electroporated with the linearized construct, and individual clones resistant to both G418 and gancyclovir were isolated and expanded for screening by Southern analysis of genomic DNA. Of 50 doubly resistant colonies screened, 12 had incorporated the predicted mutation in the *p107* gene by homologous recombination, as judged by

genomic Southern blot using a 5' probe that lies outside the region of homology with the targeting vector as well as a 3' probe (Fig. 1B).

Following injection into C57BL/6 blastocysts, two of the properly targeted ES cell clones produced chimeras which transmitted the *p107* mutation through the germline. As expected, half of the agouti offspring of such chimeras were heterozygous for the mutant *p107* allele based on Southern blot analysis of tail DNA. To determine whether mice homozygous for the disrupted *p107* gene were viable, heterozygotes were interbred and their progeny were genotyped. Southern analysis of tail DNA (see Fig. 1C) showed the expected Mendelian distribution of genotypes: of 130 animals screened, 33 were wild-type, 68 were heterozygous, and 29 were homozygous for the mutant allele. Thus, *p107* is a nonessential gene in the mouse.

p107 homozygous mutant mice do not produce detectable p107 protein and mRNA

To confirm that the *p107* gene had been fully inactivated, the level of p107 protein was examined in extracts of embryonic fibroblasts derived from wild-type and *p107* mutant mice. Extracts were immunoprecipitated with a mixture of anti-p107 monoclonal antibodies (SD9 and SD15), and the immunoprecipitates were analyzed by western blot with an anti-p107 monoclonal antibody (SD9) which recognizes an epitope in the "spacer" region of p107. As shown in Figure 2A, extracts from the homozygous mutant embryonic fibroblasts do not contain any detectable p107 protein, whereas the level of p107 protein in heterozygous mutants was approximately 50% of wild-type. Similarly, western blot analysis of extracts made from whole embryos (E18) probed with the SD9 monoclonal antibody detected no p107 protein (data not shown.). Lack of p107 in the mutant mice was further confirmed by analysis of RNA. Northern blotting of poly(A)$^+$ RNA from wild-type and *p107* mutant whole embryos (E18) demonstrated that *p107*$^{-/-}$ mice lack normal *p107* mRNA (Fig. 2B).

We also examined the possibility of compensatory up-regulation of pRB and p130 protein in the absence of p107. Western blot analysis of total proteins isolated from wild-type and mutant embryos with antibodies specific to pRB or p130 indicated that relative to wild-type, no significant difference was observed in the amount of pRB and p130 in whole mutant embryos (E18) (Fig. 2C), or separately in their liver, heart, lung, and brain (data not shown).

p107 mutant mice are viable and fertile

p107$^{+/-}$ and *p107*$^{-/-}$ mice have not displayed any increased morbidity or mortality up to 24 months of age. Routine comparative gross and histological surveys of internal organs of 4- to 12-month old homozygous mutant animals did not reveal any developmental or pathological abnormalities. The development of T- and B-cells in the thymus and spleen of mutant mice were also normal compared to wild-type, as determined by the expression pattern of CD4/CD8 and IgG/IgM, respectively (data not shown). Additionally, the loss of p107 did not impair fertility, as litter sizes from homozygous breeding pairs were similar to those of wild-type mice. Mutant mice generated on an inbred 129/Sv genetic background also did not show any abnormal phenotype up to 10 months of age. However, upon careful examination of p107$^{-/-}$ embryos, we have observed subtle thickening of the bones of the forelimbs (D. Cobrinik et al., manuscript in preparation).

p107-deficient embryonic fibroblasts

p107 has been implicated in cell cycle control, because it forms complexes with E2F and cdk2/cyclin A or cdk2/cyclin E in G_1/S phase. To assess directly the affects of p107 deficiency on cell cycle regulation, we examined the cell cycle features of p107-deficient embryonic fibroblasts (passage 4 to 6) derived from 12.5 days embryos. The p107-deficient cells did not show significant differences from wild-type cells in cell cycle profile as determined by flow cytometric analysis of BrdU/propidium iodide-labeled cells, growth curves, length of G1 phase after serum

release, or serum dependency (data not shown). These data do not support an obligatory role for p107 in cell cycle regulation, at least in this cell type.

Rb+/-;p107+/- mice

We tested the possibility that p107 and pRB might perform partially overlapping functions by crossing the *p107*-homozygous mutant mice to mice carrying the Rb^{x3t} mutation (Jacks et al., 1992). The initial cross of $p107^{-/-}$ to $Rb^{+/-}$ mice yielded $Rb^{+/-};p107^{+/-}$ offspring with the expected frequency of 50%, and the phenotype of these animals was indistinguishable from that of their $Rb^{+/-}$ littermates. $Rb^{+/-};p107^{+/-}$ animals died from tumors in the intermediate lobe of the pituitary as seen in the $Rb^{+/-}$ mice at approximately one year of age. PCR analysis of 7 pituitary tumors from these mice indicated that the wild-type allele of *Rb* was absent while the wild-type allele of *p107* was retained (data not shown.). Thus, there appears to be no selective advantage to loss of *p107* function during tumor development in this tissue.

Retarded growth of *Rb+/-;p107-/-* mice

To investigate the effect of p107 deficiency on the $Rb^{+/-}$ phenotypes, $Rb^{+/-};p107^{-/-}$ mice were generated. To generate $Rb^{+/-};p107^{-/-}$ mice, $Rb^{+/-};p107^{+/-}$ mice were intercrossed or crossed to $p107^{-/-}$ mice. As assessed at 3 - 4 weeks of age, the number of $Rb^{+/-};p107^{-/-}$ animals obtained was markedly lower than expected (Table 1A and 1B). Litters contained pups of approximately uniform size at birth, but after several days, some runted pups were evident. Genotyping showed that the runted pups were consistently $Rb^{+/-};p107^{-/-}$, and they were born at somewhat lower than the expected frequency. Of 81 pups genotyped at one week of age from $Rb^{+/-};p107^{+/-}$ by $p107^{-/-}$ crosses, 15 (19%) were $Rb^{+/-};p107^{-/-}$, which is significantly fewer than expected frequency of 25%. These mice were severely growth retarded for several weeks, averaging approximately 50% of the weight of their littermates (Fig. 3A). The average body weight of 1 week old $Rb^{+/-};p107^{-/-}$ mice was 3.2 ± 0.4 g (n= 8), whereas that of $Rb^{+/-};p107^{+/-}$ mice was 6.2 ± 0.3 g

(n= 6), which is comparable to the other mutant genotypes and wild-type. At 2 weeks of age, a similar difference persisted: 4.5 ± 1.2 g (n= 10) for *Rb*$^{+/-}$;*p107*$^{-/-}$ compared to 11.0 ± 0.6 g (n= 8) for *Rb*$^{+/-}$;*p107*$^{+/-}$. Approximately 25% of these *Rb*$^{+/-}$;*p107*$^{-/-}$ mice survived beyond three weeks of age. Most of these surviving animals gained weight slowly, reaching 70 - 90% of normal weight after 3 months. A representative growth curve for *Rb*$^{+/-}$;*p107*$^{-/-}$ mice is shown in Figure 3B. Most of the male *Rb*$^{+/-}$;*p107*$^{-/-}$ animals were fertile (Table 1C and 1D) and apparently normal up to 12 months of age. However, approximately 75% of female *Rb*$^{+/-}$;*p107*$^{-/-}$ mice had vaginal atresia (Fig. 3C). Although the vaginal opening was not present in the affected females, the remainder of the reproductive tract appeared normal (not shown). Between 4 - 6 months of age, the affected female mice accumulated fluid in the uterus and died. Surviving *Rb*$^{+/-}$;*p107*$^{-/-}$ mice subsequently succumbed to pituitary tumors associated with their *Rb*$^{+/-}$ status after 12 months. Thus, at least out to one year of age, there is no obvious additional tumor phenotype associated with this mutant combination.

Retinal dysplasia in *Rb+/-;p107-/-* mice

Although retinoblastoma does not develop in *Rb*$^{+/-}$ mice or chimeric mice constructed with *Rb*$^{-/-}$ ES cells (Williams et al., 1994; Maandag et al., 1994), this tumor is observed upon retinal expression of viral oncogenes that bind and inactivate all three members of the pRB family. Therefore, we carefully examined the retinas of surviving *Rb*$^{+/-}$;*p107*$^{-/-}$ animals at the age of 4 - 6 months by indirect stereoscopic ophthalmoscopy. Retinal lesions were detected in all 12 *Rb*$^{+/-}$;*p107*$^{-/-}$ mice examined, and 11 out of the 12 were affected in both eyes. Typically, each eye exhibited 5 to 7 individual lesions consisting of small white bodies, usually in clusters (Figure 4A). No retinal abnormalities were detected in any of 8 *p107*$^{-/-}$ mice examined or in greater than 20 *Rb*$^{+/-}$ mice examined (data not shown). Histological analysis of eyes from *Rb*$^{+/-}$;*p107*$^{-/-}$ mice confirmed the presence of retinal lesions, which were characterized as severe retinal dysplasia or disruption of the normal photoreceptor architecture (Figs. 4B to 4E). Histological analysis of retinas taken from 4 day old *Rb*$^{+/-}$;*p107*$^{-/-}$ neonates also showed the presence of subtle, yet

significant disruptions of the photoreceptor layer of the retina (Figs. 4F and 4G), which could represent precursors to retinal dysplasia.

Early death of *Rb-/-;p107-/-* embryos

pRB is required for normal mouse development (Lee et al., 1992, Jacks et al., 1992, Clarke et al, 1992). $Rb^{-/-}$ embryos die between 13.5 - 15.5 days of gestation, with excessive apoptotic cell death and inefficient erythroid differentiation. In order to examine the developmental consequences of inactivation of both *Rb* and *p107*, $Rb^{+/-};p107^{-/-}$ males were crossed to $Rb^{+/-};p107^{+/-}$ females to produce double mutant embryos at an expected frequency of 12.5%. Embryos were recovered at different days of gestation, examined morphologically and genotyped. As shown in Figure 5, in contrast to $Rb^{-/-}$ embryos, the double mutant embryos died significantly earlier than 13.5 days of gestation. Analysis of E11.5 embryos revealed that of the 19 $Rb^{-/-};p107^{-/-}$ embryos examined, 9 were dead and 2 were noticeably smaller than their littermates. Histological analysis of the surviving embryos revealed an abnormally high frequency of pyknotic nuclei (characteristic of apoptosis) in the central nervous system (CNS) (Fig. 6B). This is in contrast to embryos deficient for pRB alone in which apoptosis in the CNS is not apparent to this extent until E13.5 (Lee et al., 1992, Jacks et al., 1992, Clarke et al, 1992). At E12.5, seven out of eight $Rb^{-/-};p107^{-/-}$ embryos detected at this stage were dead or in various stages of resorption. Detailed histological analysis of the viable embryo revealed abnormally high levels of apoptosis in the liver as indicated by the presence of pyknotic nuclei (Fig. 6D) and in the CNS (data not shown). $Rb^{-/-}$ embryos did not show this degree of apoptosis in the liver at this developmental stage (Fig. 6C). The apoptotic cell death in the liver of double mutants was not evident at E11.5. These data indicate that the p107 deficiency enhances the induction of apoptosis associated with loss of pRB in the liver and CNS.

Analysis of E10.5 embryos showed that of 10 double mutant embryos recovered, 2 were dead and 2 were noticeably smaller than the majority of their control littermates. However, histological

analysis of the viable embryos did not reveal any obvious pathology at this developmental stage. In an effort to elucidate the cause of early death of *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos, histological analysis of yolk sacs from viable double mutant embryos at 11.5 days of gestation was performed. Compared to *Rb*$^{+/-}$;*p107*$^{-/-}$ embryos, there was a significant decrease in the number of erythrocytes in the yolk sac blood vessels as well as disorganization of the endothelial linings of the vessels. However, *Rb*$^{-/-}$ mice also frequently showed a similar defect at the same gestational age (data not shown; Clarke et al., 1992). Therefore, it remains unclear why the *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos fail earlier in gestation than *Rb*$^{-/-}$ embryos.

Muscle formation of *Rb-/-;p107-/-* embryos

The expression of viral oncoproteins that bind to pRB, p107, and p130 induces pleiotropic effects, including the inhibition of myogenic differentiation (Webster et al., 1988; Endo 1992; Maione et al., 1992) and induction of DNA synthesis in the nuclei of differentiated myotubes (Endo 1992). Although aberrant cell cycle re-entry has been reported in *Rb*$^{-/-}$ myotubes (Schneider et al., 1994), most aspects of skeletal muscle differentiation are observed in pRB-deficient embryos (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992) and cells (Schneider et al., 1994). In light of the partially overlapping developmental functions of the *Rb* gene family (this study and D. Cobrinik et al., manuscript in preparation), as well as previous suggestions of functional redundancy between pRB and p107 in muscle development (Gu et al., 1993; Schneider et al., 1994), we examined *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos for the presence of skeletal muscle. Histological analysis of double mutant and control embryos at E12.5 revealed myofibers in the body wall and primordial tongue in all embryos (data not shown). In addition, immunohistochemical analysis with an antibody specific for sarcomeric actin showed comparable staining in the body wall of *Rb*$^{-/-}$;*p107*$^{-/-}$ (Fig. 6F) and control embryos (Fig. 6E). This indicates that at this stage in development, both myogenic commitment and differentiation can occur normally in the absence of both pRB and p107.

Discussion

pRB, p107, and p130 comprise a family of proteins sharing common structural organization and a significant degree of sequence similarity. Evidence from many different experimental systems has suggested that this family of proteins has critical functions in the control of cell proliferation and differentiation. In the present study, we have addressed the biological role of *p107* and its functional relationship with *Rb*. Using gene targeting in ES cells, we have generated p107-deficient mice. Unexpectedly, these mice are viable, healthy, and fertile. The oldest $p107^{-/-}$ mice are now greater than 2 years of age, and to date, no tumor phenotype is evident. Furthermore, the analysis of embryonic fibroblasts derived from p107-deficient embryos did not reveal any detectable differences in cell cycle characteristics relative to wild-type cells.

These findings are surprising in light of the extensive literature describing studies carried out in tissue culture cells indicating that p107 may function in control of the cell cycle progression, in the regulation of cell proliferation and cell differentiation (See for example, Cobrinik et al., 1993; Schwarz et al., 1993; Zhu et al., 1993; Beijersbergen et al., 1994a; Beijersbergen et al., 1994b; Gu et al., 1994; Kim et al., 1994; Schneider, et al., 1994; Hoang et al., 1995). The absence of overt abnormalities in p107-deficient cells or p107-deficient mice would on the surface appear to contradict these previous findings. It may be that p107 function is not essential in these processes and that the earlier results derive from ectopic *p107* overexpression. However, it is also possible that p107 is normally involved in cell cycle progression and differentiation, but that in its absence, other proteins, including other members of the *Rb* gene family can functionally compensate for its absence.

The data presented in this paper shows that *p107* and *Rb* have overlapping function in some tissues at specific stages of development. Embryos deficient for pRB die between 13.5 and 15.5 days of gestation. In contrast, $Rb^{-/-};p107^{-/-}$ embryos died beginning at 10.5 days of gestation with none surviving beyond 12.5 days. Thus, a requirement for p107 function in mid-gestation

development is revealed in the context of pRB deficiency. The early death of the double mutant embryos was accompanied by accelerated apoptotic cell death in the liver and central nervous system compared to *Rb* mutant embryos alone. It is noteworthy that mice deficient for p130 or both p107 and p130 did not show embryonic lethality or obvious abnormality in apoptotic cell death (D. Cobrinik et al., manuscript in preparation); however, p107 and p130 share overlapping functions in other cell types and at other stages of development.

The functional overlap between *p107* and *Rb* could occur at several different levels. At one extreme, the enhanced phenotypes of the *Rb/p107* double mutnat mice might be due to true biochemical redundancy. Here, the redundancy might arise from naturally shared activities or might be fostered by the loss of a closely related family member. At the other extreme, functional overlap might arise from the combination of partially crippled pathways that when brought together in the double mutants greatly magnifies the phenotype in the affected cell. Whichever the case, the novel phenotypes described here will provide the needed materials to decipher the overlapping activities of these important regulators of proliferation.

Since $Rb^{-/-};p107^{-/-}$ embryos showed an altered phenotype, we investigated whether loss of p107 affected the tumor phenotype of $Rb^{+/-}$ mice. $Rb^{+/-};p107^{-/-}$ mice showed severe growth retardation but did not display any additional tumor phenotype beyond the pituitary tumors caused by *Rb* heterozygosity. However, $Rb^{+/-};p107^{-/-}$ mice developed focal lesions in the retina with degeneration of the photoreceptor layer and retinal dysplasia. Such lesions are not present in $Rb^{+/-}$ or $p107^{-/-}$ mice. Moreover, lesions of this type were not found in chimeric mice partly composed of $Rb^{-/-}$ cells (Maandag et al., 1994). Although the frequency and focal nature of these lesions suggests that they result from the inactivation of the remaining wild-type *Rb* allele, this has not yet been demonstrated experimentally. The defect in the photoreceptor layer is reminiscent of the degeneration of the retina due to photoreceptor cell death in transgenic mice following the expression of the HPV-16 E7 oncogene exclusively in photoreceptor cells and pineal gland (Howes et al., 1994). However, as the E7 protein can bind to pRB, p107, and p130, it was unclear

from these studies whether the cell proliferation and apoptosis caused by E7 expression resulted from the inactivation of all three proteins or from inactivation of specific family members. In the present study, retinal lesions were not found in *p130*$^{-/-}$, *Rb*$^{+/-}$;*p130*$^{-/-}$, *p107*$^{+/-}$;*p130*$^{-/-}$, and *p107*$^{-/-}$;*p130*$^{+/-}$ mice (unpublished data), suggesting that degeneration of the retinal layer may be a specific consequence of the inactivation of *Rb* and *p107*. These data suggest that the entire photoreceptor layer would be affected in *Rb*$^{-/-}$;*p107*$^{-/-}$ mice. However, such an analysis is precluded by the fact that double mutant embryos die prior to retinal differentiation. The production of chimeric mice using ES cells mutant for both *Rb* and *p107* may address this question.

Although mutation of the *p107* gene is unlikely to be a rate-limiting or initiating event for tumor formation, the appearance of the retinal lesions in *Rb*$^{+/-}$;*p107*$^{-/-}$ mice suggests that there may be specific settings in which mutation of *p107* may promote tumor progression. By analogy with the cooperative effects of HPV-16 E6 and E7 in tumorigenesis in the retina (Howes et al., 1994), one might expect that germline mutation of *p53* along with *Rb* and *p107* might lead to frank retinoblastoma. Further studies are needed to identify additional genetic backgrounds in which loss of p107 function might contribute to tumorigenesis.

Taken together these observations are consistent with the notion that p107 and pRB can serve related functions in growth control and/or differentiation in some developmental contexts but not in the inhibition of tumor formation, save perhaps for the retina. Since p107 and pRB both interact with E2F but associate with different subsets of E2F polypeptides (Beijersbergen et al., 1994; Ginsberg et al., 1994), it is possible that their roles in the regulation of E2F may explain both the specific and related functions. Alternatively, other growth control and differentiation pathways normally regulated by these proteins may contribute to the mutant phenotypes. Further characterization of the biochemical basis of the observed defects should help elucidate the relevant pathways.

Materials and methods

Isolation of genomic *p107* clones

A mouse genomic library constructed from the liver DNA of the strain 129/Sv (Stratagene) was screened using a fragment of the mouse p107 cDNA as a probe. A 350-nucleotide fragment from the 5' end region containing the ATG translation initiation codon was prepared by PCR and was radiolabeled by random primer labeling techniques (Pharmacia). Filters were washed under high stringency (0.5 x SSC, 0.1% SDS at 65°C). Several clones were isolated, and a lambda clone with a 15 kb genomic insert was identified as containing exon 1 by Southern blot analysis and sequencing. The insert was subcloned into pBluescript SK (Stratagene) and extensively mapped with restriction enzymes.

Construction of the targeting vector

The targeting vector was derived from the pPNT vector, which contains the thymidine kinase gene and the neomycin resistance gene (Tybulewicz et al., 1991). The 4.0 kb Pst1 mouse genomic fragment and 3.0 kb HindIII-Not1 (derived from a cloning site of pBluescript SK) fragment surrounding the first exon of the mouse *p107* gene (which encodes amino acids 1 - 52) were cloned into pPNT surrounding the neomycin resistance gene. The unique Not1 site in the construct was used to linearize the plasmid prior to electroporation into ES cells.

ES cell culture, transfection, and selection

The D3 ES cell line (passage 5), derived from strain 129/Sv, was grown on feeder layers of g-irradiated (1500 rad for 30 min) embryonic fibroblasts in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated fetal calf serum (Hyclone), 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 1000 units/ml mouse leukemia inhibitory factor (ESGRO; GIBCO BRL). Cells (8×10^6) were electroporated in 0.8 ml of medium with 40 mg of linearized targeting vector DNA at 400 V/250 mF in a Bio-Rad Gene Pulser, and were plated on feeder cells. Selection with 300 mg/ml G418 (GIBCO BRL) and 2 mM gancyclovir was started 48 hr later. Three hundred doubly resistant colonies were selected for screening by Southern analysis.

Southern blot analysis to identify targeted clones

Individual ES cell clones were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% SDS, and 0.25 mg/ml proteinase K overnight at 37°C. Samples were extracted with phenol-chloroform, and genomic DNA was isolated by ethanol precipitation. Genomic DNA (10 mg) from each clone was digested with Hpa1 and Sph1, resolved by electrophoresis on 0.9% agarose gels, and transferred to nylon membrane (Hybond-N^+; Amersham). Prehybridization was carried out in 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% bovine serum albumin (BSA), and 7% SDS at 65°C. Hybridization was performed with the ^{32}P-labeled probe in 0.2 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, 15% formamide, and 100 mg/ml salmon sperm DNA at 65°C. The 5' hybridization probe was a 950 bp Rsa1 fragment derived from 1.5 kb Hpa1-EcoR1 fragment of p107 genomic DNA, which contained sequences not included in the targeting vector. A 500 bp HaeIII fragment derived from a 1 kb EcoR1-Sph1 fragment was used as the 3' probe. Filters were washed three times in washing buffer (40 mM sodium phosphate, pH 7.2, 1 mM EDTA, and 1% SDS) at 65°C, and exposed to film.

Generation and breeding of chimeric mice

Blastocysts were isolated from C57BL/6 mice at day 3.5 of pregnancy, and 12 ES cells were injected into the blastocoel cavity. The blastocysts were implanted into the uterine horns of pseudopregnant CD1 recipient females. Chimeric offspring were identified by agouti contribution to the coat color due to the ES cells and were crossed to C57BL/6 females. Germ-line transmission of the mutant allele was assessed by Southern blot analysis of tail biopsies of agouti progeny.

PCR analysis of *p107* genotype

DNA from tail biopsies or yolk sacs was isolated as described for the Southern blot analysis above. A 330 bp PCR fragment specific for the *p107* mutation allele was amplified using primers 3' to the disrupted region ($p107_{com}$ = 5'-TCGCTGGCAGTCTGAGTCAG-3') and within the integrated *neo* construct ($p107_{neo}$ = 5'-ACGAGACTAGTGAGACGTGC-3'). A 280 bp PCR fragment specific for the wild-type *p107* allele was amplified using the same $p107_{com}$ primer used above and a primer corresponding to the region deleted in the mutant allele ($p107_{wt}$ = 5'-TGTCCTGAGCATGAACAGAC-3'). DNA samples were amplified in 20 ml reaction in buffer containing 60 mM Tris-HCl, pH 9.0, 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 1mM of each dNTP, 1 unit Taq polymerase (Promega), and 1 mM of each primer. Samples were amplified for 30 cycles for 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Primers for PCR analysis of *Rb* genotype and reaction conditions for amplification have been described previously (Jacks et al., 1992).

Northern and western blot analyses

For northern blot analysis, total RNA was isolated from embryos by guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchhi, 1987) and enriched for poly(A)$^+$ RNA using oligo(dT) cellulose. Samples (4 mg) were resolved by electrophoresis on 1% agarose gels

containing formaldehyde, transferred to nylon membrane (Hybond-N^+; Amersham), hybridized to a 4.8 kb mouse full-length *p107* cDNA probe in the presence of 30% formamide, with other conditions as described above for Southern blotting. To prepare cell lysates, cells were extracted with lysis buffer (50 mM HEPES buffer, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfony fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 0.5 mM DTT, and 0.5 mM sodium fluoride), and centrifuged for 10,000 x g for 10 min. Embryos were frozen and pulverized in liquid nitrogen before extraction. Protein concentration was measured with a Coomassie blue dye binding assay (Bio-Rad). To immunoprecipitate p107 in lysates, the supernatants were incubated with a mixture of monoclonal anti-p107 antibodies (SD9 and SD15) at 4°C for 1 hour. For western blot analysis, the immunoprecipitates or cell lysates were denatured in SDS loading buffer and separated by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with anti-p107 antibody (SD9) or monoclonal anti-pRB antibody (3C8, Kanji), or polyclonal anti-p130 antibody (C-20, Santa Cruz), and then subsequently incubated with a goat anti-rabbit or anti-mouse horseradish peroxidase-labeled antibody (Amersham). Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham).

Retinal examination by indirect ophthalmoscopy and fundus photography

Pupils were dilated by topically applying eye drops consisting of 2.5% phenylephrine-HCl and 0.4% tropicamide (Phen/Trop, Mass Eye and Ear Infirmary Formulary, diluted 1:1 with sterile BSS) to the cornea of each eye. The pupils dilated fully within 5 minutes. The retina was examined *in situ* with a standard, clinical indirect ophthalmoscope (Keeler Fision) using a 60, 78, or 90 diopter condensing lens (Volk, Nikon) depending on the desired magnification and field of view. Fundus photographs were taken using a Kowa RC-2 hand-held fundus camera and a 60 or 78 diopter condensing lens on Kodak Ektachrome 100 Professional color reversal film (S. Mukai, manuscripts in preparation).

Histology and immunohistochemistry

For histological analyses, tissues were removed, fixed in phosphate-buffered 10% formalin, dehydrated in increasing concentrations of ethanol followed by xylene, embedded in paraffin, sectioned at 6 mm, and stained with haematoxylin and eosin. For immunohistochemistry, embryo sections were deparaffinized and rehydrated before a 20 minute incubation in block solution: 2% goat serum, 1% horse serum, 0.1% Triton X-100 in PBS. Sections were then incubated for 1 hour with anti-sarcomeric actin monoclonal antibody (Sigma) diluted 1:1,000 in block. After 3 washes in PBS, antibody was detected with Vecta Stain Elite ABC Peroxidase Kit (Vector Laboratories) and counterstained with 0.25% methyl green.

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Table 1. Offspring viability of *Rb+/-;p107-/-* mice at 3 - 4 weeks of age

A. $Rb^{+/-};p107^{+/-}$ x $Rb^{+/-};p107^{+/-}$

Genotype[a] *Rb*	+/+	+/+	+/+	+/-	+/-	+/-
p107	+/+	+/-	-/-	+/+	+/-	-/-
expected ratio	0.5	1	1	1	2	1
numbers obtained	4 (0.4)	10 (1.0)	9 (0.9)	14 (1.4)	29 (2.9)	3(0.3)

B. $Rb^{+/-};p107^{+/-}$ x $p107^{-/-}$

Genotype *Rb*	+/+	+/+	+/-	+/-
p107	+/-	-/-	+/-	-/-
expected ratio	1	1	1	1
numbers obtained	148 (1.0)	179 (1.2)	157 (1.1)	38 (0.26)

C. $Rb^{+/-};p107^{-/-}$, male x $p107^{-/-}$, female

Genotype *Rb*	+/+	+/-
p107	-/-	-/-
expected ratio	1	1
numbers obtained	69 (1.0)	14 (0.2)

D. $Rb^{+/-};p107^{-/-}$, male x $Rb^{+/-};p107^{+/-}$, female

Genotype[a] *Rb*	+/+	+/+	+/-	+/-
p107	+/-	-/-	+/-	-/-
expected ratio	1	1	2	2
numbers obtained	16 (1.0)	16 (1.0)	42 (2.6)	5 (0.3)

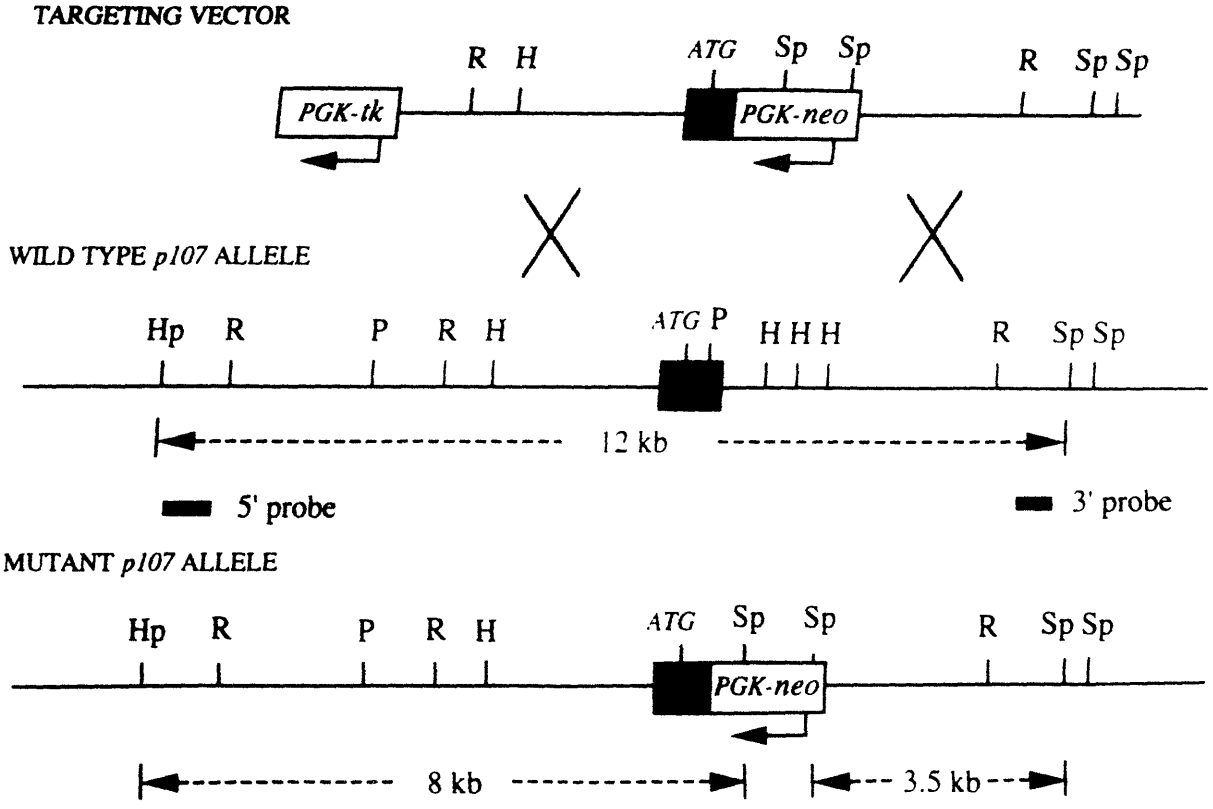
Genotype analysis was performed using DNA prepared from tail biopsies of 3 - 4 weeks old live offspring from each cross. The number of $Rb^{+/+};p107^{+/-}$ mice or $Rb^{+/+};p107^{-/-}$ mice was arbitrarily assigned a value of 1.0 and relative ratios of the other genotypes were calculated accordingly (numbers in parentheses).

[a] Several genotypes of mice that are embryonic lethal due to an $Rb^{-/-}$ genotype were excluded.

Figure Legends

Figure 1. Targeted disruption of the mouse *p107* gene. (A) The restriction map of targeting vector, wild-type, and mutant *p107* alleles. Homologous recombination would result in replacement of 2.3 kb fragment from the *p107* gene with a PGK-*neo* expression cassette. The arrows indicate the direction of transcription of PGK-*neo* and PGK-*tk* expression cassette, which are in the opposite direction to *p107*. A closed box represents *p107* exon sequence containing the first ATG translation initiation sequence. *p107* gene 5' flanking probe and 3' probe used for screening of ES cell clones and mutant mice are indicated, together with the expected size of hybridizing restriction fragments resulting from a double digestion with Hpa1 and Sph1 for the wild-type and mutant alleles. R, EcoR1; P, Pst1; H, HindIII; Hp, Hpa1; Sp, Sph1. (B) Southern blot analysis of Hpa1 plus Sph1-digested genomic DNA from ES cells. Hybridizing fragments with the 5' probe are 12 kb for the wild-type allele (wt) and 8 kb for the mutant allele (m), and with the 3' probe, 12 kb for wild-type allele (wt) and 3.5 kb for the mutant allele (m). (C) A representative Southern blot analysis of progeny from a heterozygous cross. Tail DNA was digested with Hpa1 and Sph1 and probed with the 5' probe. The genotypes are depicted at the top of each lane.

A


TARGETING VECTOR
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PGK-tk
PGK-neo
WILD TYPE p107 ALLELE
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ATG P
H H H
R Sp Sp
12 kb
5' probe
3' probe
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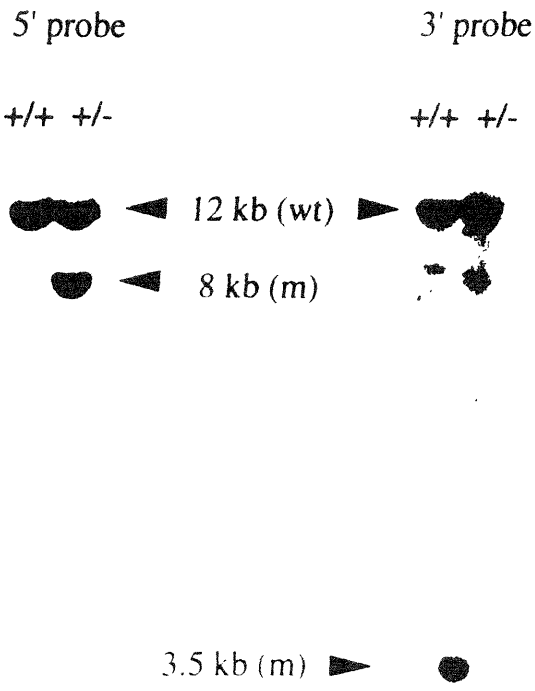
PGK-neo
8 kb
3.5 kb

B



C

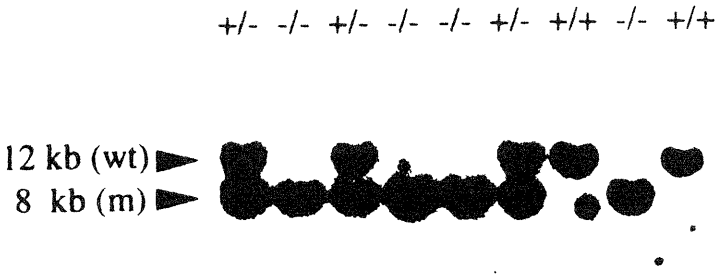
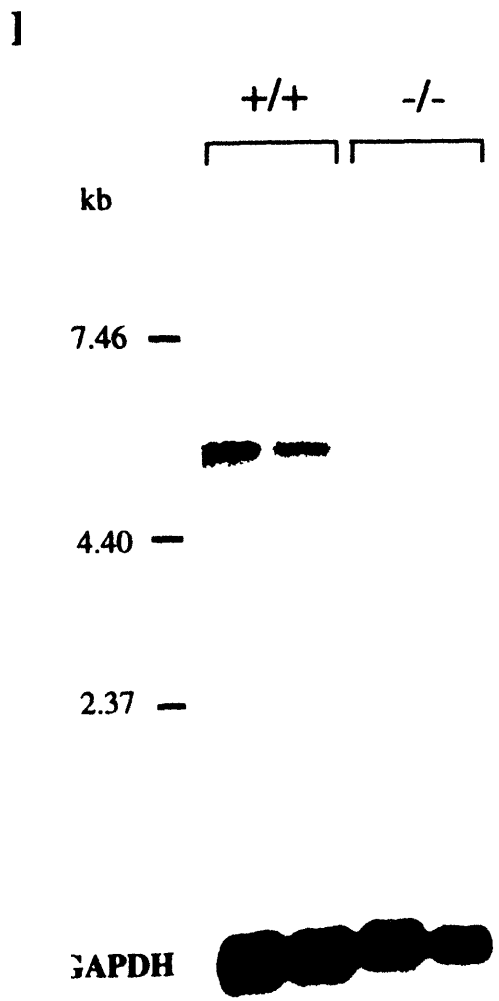
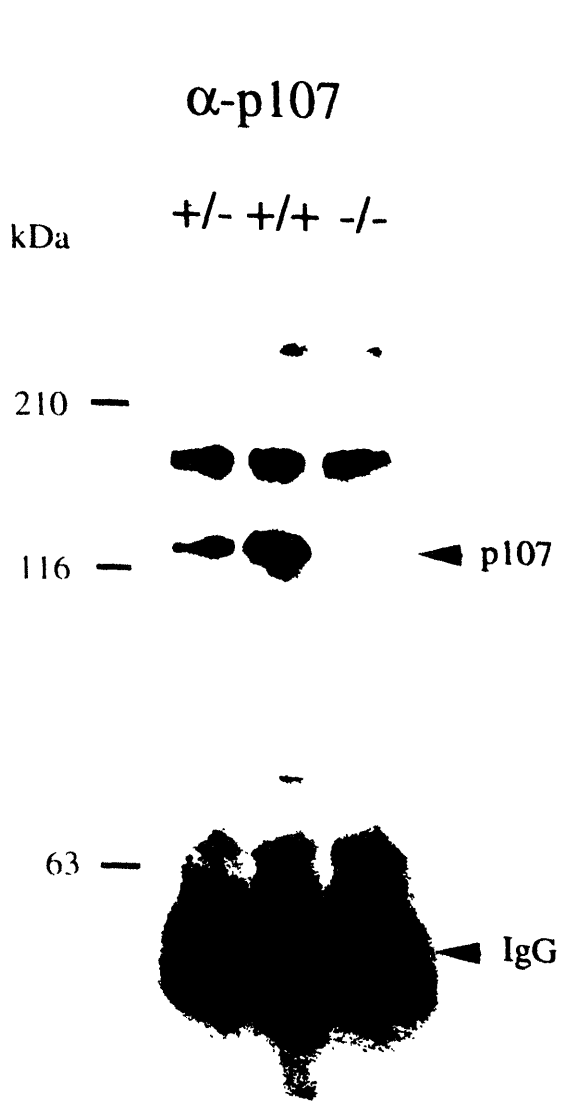


Figure 2. Western blot and Northern blot analysis of *p107* mutant mice and cells. (A) Western blot analysis of cell extracts prepared from wild-type (+/+), $p107^{+/-}$, and $p107^{-/-}$ embryos MEFs using a monoclonal antibody against to p107. Cell extracts were immunoprecipitated with monoclonal antibodies against p107 (SD9 and SD15) and the p107 immunoreactive species were detected by immunoblotting with SD9. Note the absence of p107 in the -/- sample and the reduced levels in the +/- sample. Positions of molecular weight standards are indicated at left The position of p107 and cross-reactive IgG is also shown at right. (B) Northern blot analysis of poly(A)$^+$ RNA isolated from wild-type and $p107^{-/-}$ whole embryos (E18), using a 4.8 kb full-length mouse p107 cDNA probe. Note the absence of *p107* transcript in the *p107* mutant (-/-) samples. The positions of RNA molecular weight markers are indicated to the left. The lower panel shows hybridization with a GAPDH probe as a loading control. (C) Western blot analysis of protein extracts from wild-type (+/+), $p107^{+/-}$, and $p107^{-/-}$ embryos (E18), using antibodies against pRB (upper blot) and p130 (lower blot). Note that pRB and p130 levels are not altered significantly in *p107*-mutant embryos.


+/+
-/-
kb
7.46
4.40
2.37
GAPDH


A
α-p107
+/- +/+ -/-
kDa
210
116
p107
63
IgG

C

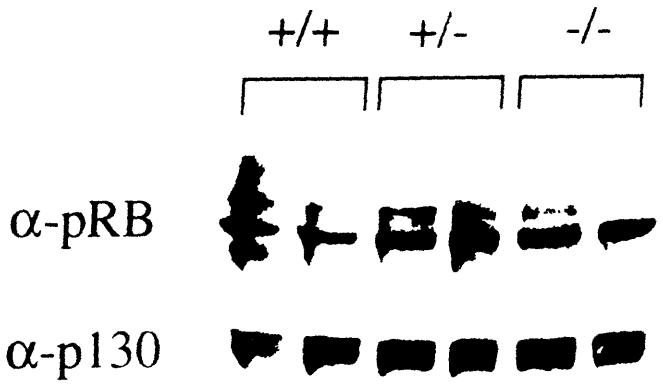
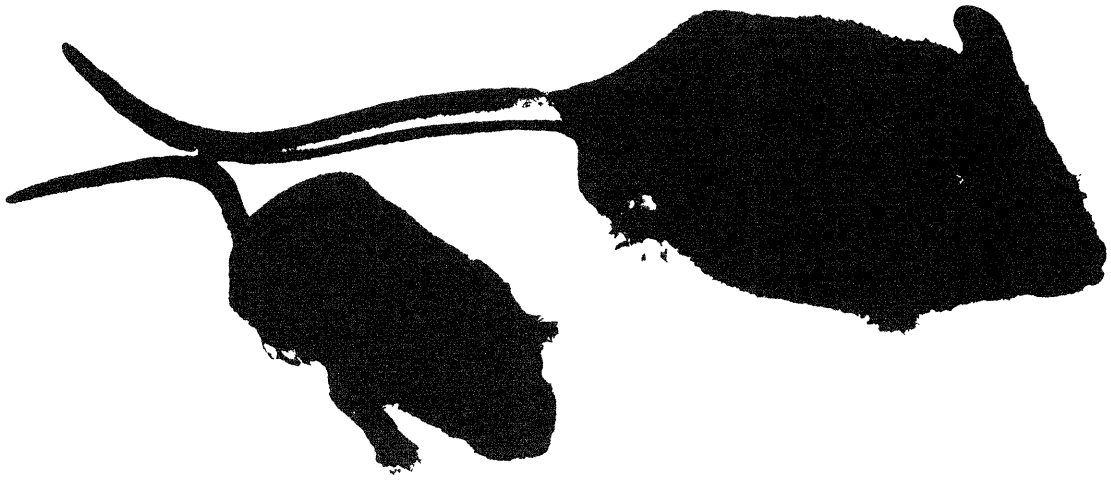


Figure 3. Phenotypes of $Rb^{+/-};p107^{-/-}$ mice. (A) Growth retardation of $Rb^{+/-};p107^{-/-}$ mice. Two week old littermates from a $Rb^{+/-};p107^{+/-}$ by $p107^{-/-}$ cross are shown. The animal at left is $Rb^{+/-};p107^{-/-}$ and is significantly growth retarded compared to the $Rb^{+/-};p107^{+/-}$ at right, as well as to wild-type mice of this age. (B) A representative growth curve of two male $Rb^{+/-};p107^{-/-}$ and two male $Rb^{+/-};p107^{+/-}$ littermates derived from a $Rb^{+/-};p107^{-/-}$ by $Rb^{+/-};p107^{+/-}$ cross. (C) Vaginal atresia in $Rb^{+/-};p107^{-/-}$ females. Note that the abdomen and perineum are distended due to fluid accumulation in the uterus.

A



C

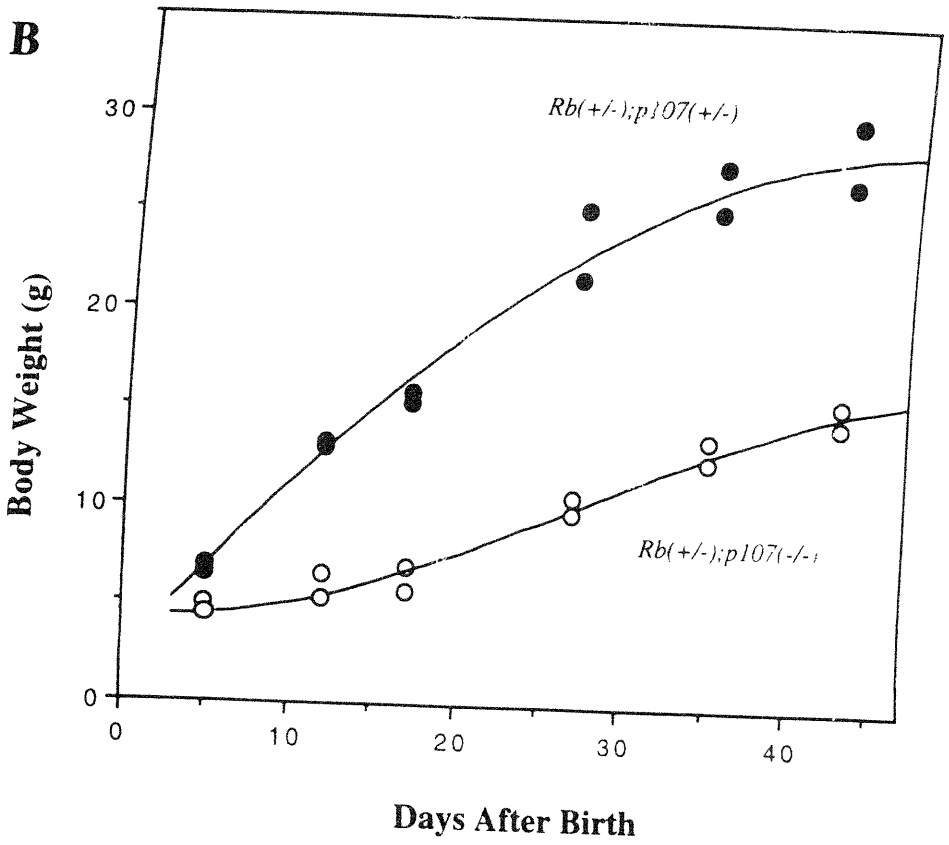

B
Rb(+/-);p107(+/-)
Rb(+/-);p107(-/-)
Body Weight (g)
0
10
20
30
0
10
20
30
40
Days After Birth

Figure 4. Retinal lesions in *Rb*$^{+/-}$;*p107*$^{-/-}$ mice. (A) Indirect ophthalmoscopy of *Rb*$^{+/-}$;*p107*$^{-/-}$ retina. White spots (solid arrows) are diagnostic of retinal pathology. One spot at the center and the crescent-shaped spot at the periphery (open arrows) are due to reflections from the camera light source. (B - C) Histological sections of retinas of *Rb*$^{+/-}$;*p107*$^{-/-}$ adult mice showing regions of retinal dysplasia (arrows); normal retinal structure is also indicated (N). (D) Higher power magnification of lesion shown in (B). (E - F) Histological sections of retinas of 4 day old *Rb*$^{+/-}$;*p107*$^{-/-}$ animals. A region of abnormal (arrow) and normal (N) retinal architecture is indicated in E. Disruption of photoreceptor layer is apparent in F. Magnifications: B, C, and E = 100 X; D and F = 400 X.

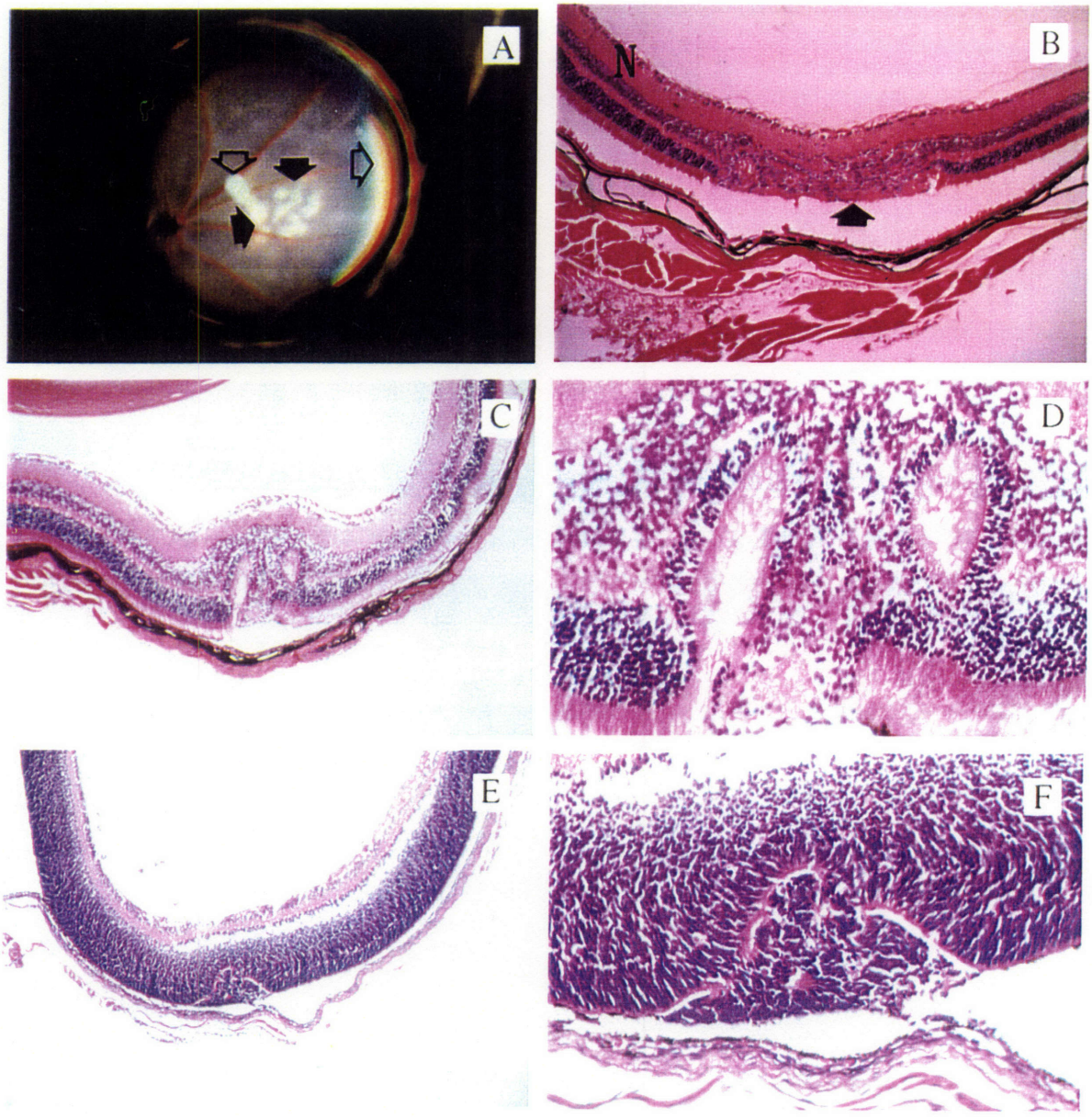

A
N
B
C
D
E
F

Figure 5. Viability of *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos. Survival as a function of gestational age of *Rb*$^{-/-}$;*p107*$^{-/-}$, *Rb*$^{-/-}$;*p107*$^{+/-}$, and control embryos. Survival percentage was determined by comparing the number of embryos with a detectable heartbeat to the total number of embryos collected at each gestational day (shown in parentheses). Embryos were derived from crosses of *Rb*$^{+/-}$;*p107*$^{-/-}$ male mice to *Rb*$^{+/-}$;*p107*$^{+/-}$ female mice. *All others* indicates the genotypes of *Rb(+/+);p107*$^{+/-}$, *Rb(+/+);p107*$^{-/-}$, *Rb*$^{+/-}$;*p107*$^{+/-}$, and *Rb*$^{+/-}$;*p107*$^{-/-}$. In addition, 100% (22/22) of *Rb*$^{-/-}$ embryo were recovered alive at E12.5.

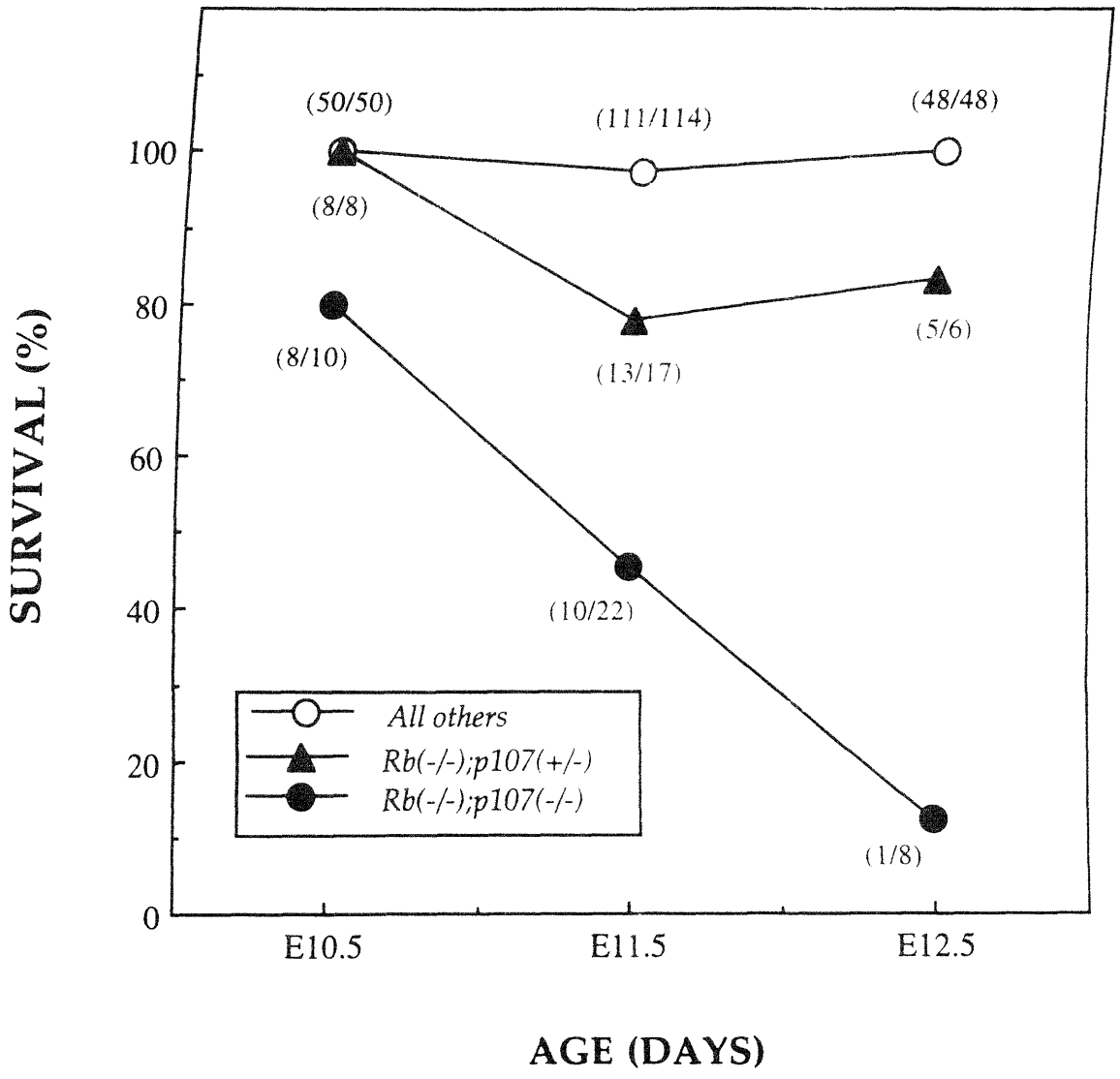
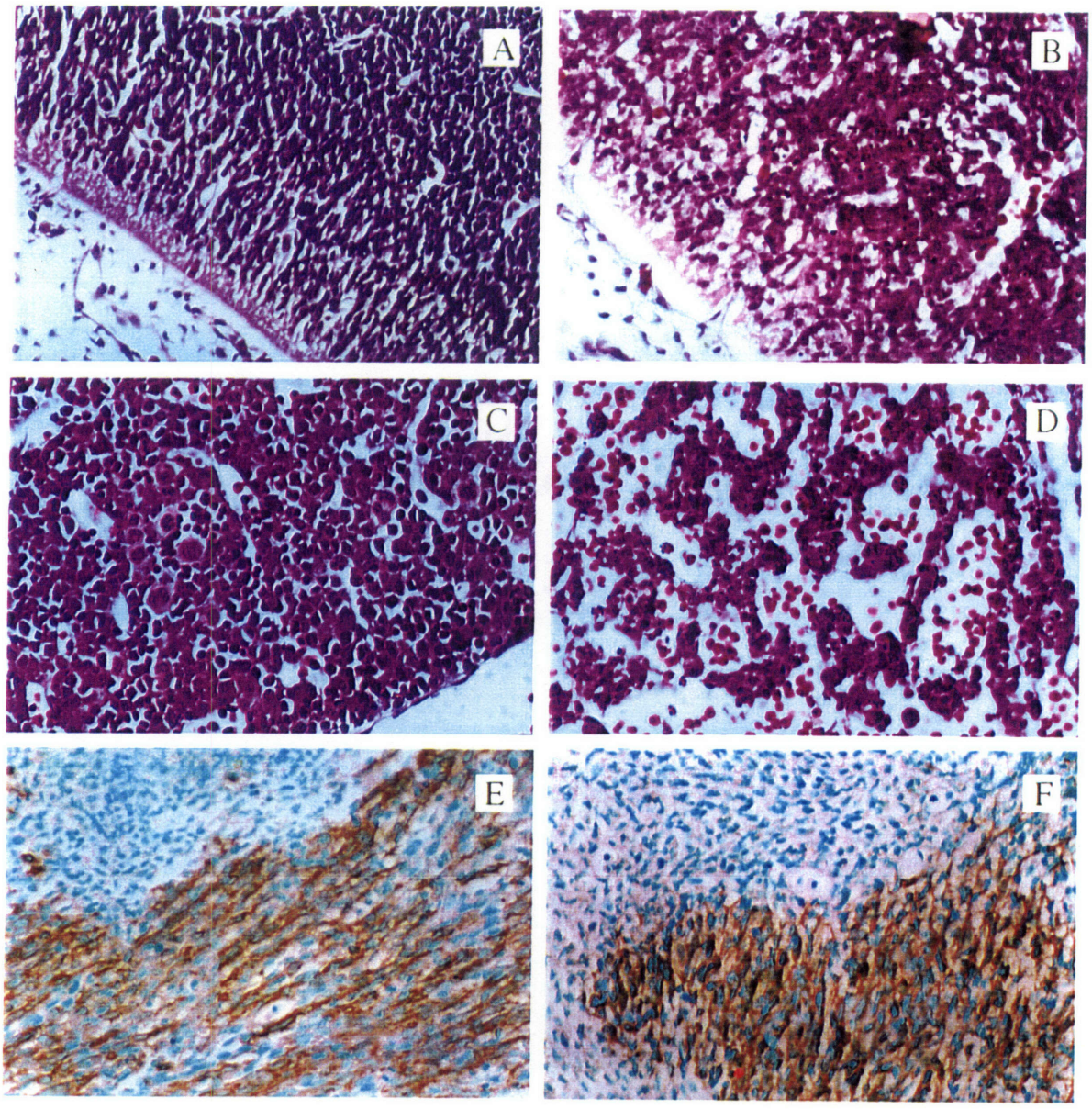

SURVIVAL (%)
100
80
60
40
20
0
(50/50)
(111/114)
(48/48)
(8/8)
(5/6)
(8/10)
(13/17)
(10/22)
(1/8)
All others
Rb(-/-);p107(+/-)
Rb(-/-);p107(-/-)
E10.5
E11.5
E12.5
AGE (DAYS)

Figure 6. Histopathology of *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos. (A and B) Sections of spinal cord from *Rb*$^{-/-}$ (A) and *Rb*$^{-/-}$;*p107*$^{-/-}$ (B) embryos at E11.5. Note that compared to the normal histology in A, the *Rb*$^{-/-}$;*p107*$^{-/-}$ tissue contains areas of acellularity and numerous cells with pyknotic nuclei indicative of apoptosis. Sections of dorsal root ganglia and peripheral nerves at this stage show apoptotic cells in both *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos (data not shown). (C and D) Liver sections from *Rb*$^{-/-}$ (C) and *Rb*$^{-/-}$;*p107*$^{-/-}$ (D) embryos at E12.5. The *Rb*$^{-/-}$;*p107*$^{-/-}$ section show areas of acellularity and numerous pyknotic nuclei, while the *Rb*$^{-/-}$ liver is normal at this stage of development. (E and F) Immunohistochemical analysis of skeletal muscle in control, *Rb*$^{+/-}$;*p107*$^{+/-}$ (E) and *Rb*$^{-/-}$;*p107*$^{-/-}$ (F) embryos at E12.5 The primary antibody is specific for sarcomeric actin; reactivity is indicated by brown precipitate. Magnifications: A - F = 100 X.


A
B
C
D
E
F

This work illustrates that one of the reasons that *Rb*-deficient embryos progress so far in development is perhaps due to the redundancy provided by the presence of the related p107 protein. Interestingly, embryos deficient for both *Rb* and *p130* fail at a similar stage in development as the *Rb-/-;p107-/-* embyros (George Mulligan and Tyler Jacks, unpublished data), suggesting that *p130* may also act in a similar way to *p107* to partially compensate for the absence of *Rb*. An analysis of embyros triply deficient for *Rb*, *p107*, and *p130* is hampered by the decreased viability and fertility of animals carrying different combinations of mutations, so an alternative route to answer this question is being pursued (see Appendix E).

The observation of retinal dysplasia in *Rb+/-;p107-/-* mice is of special note. Since retinal dysplasia has not been observed in either *Rb+/-* or *p107 -/-* mice, some aspect of retinal differentiation appears to depend on redundant functions of *Rb* and *p107*. In addition, *Rb+/-;p53-/-* mice also develop retinal dysplasia. Based on these two observations, it is tempting to speculate that the induction of retinoblastoma in mice by retinally-directed expression of SV40 Large T antigen (Windle et al., 1990) or by concomitant retinal expression of HPV E6 and E7 (Howes et al., 1994) is caused by the inactivation of *Rb*, *p107*, and *p53*. Perhaps the generation of ES cells deficient for *Rb*, *p107*, and *p53* and the subsequent generation of chimeras from them will result in the formation of retinoblastoma and resolve the question of what genetic changes are needed to induce murine retinoblastoma .

Literature Cited

Additional references can be found within this appendix on pages 233-236.

Cobrinik D. (1996). Regulatory interactions among E2Fs and cell cycle control proteins. Curr Top Microbiol Immunol. 208: 31-61.

Howes KA, Ransom N, Papermaster DS, Lasudry JGH. Albert DM and Windle JJ. (1994). Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 *E7* gene in the presence or absence of *p53*. Genes Dev. 8: 1300-1310.

Windle JJ, Albert DA, O'Brien JM, Marcus DM, Disteche CM, Bernards R and Mellon PL. (1990). Retinoblastoma in transgenic mice. Nature. 343: 665-668.

APPENDIX D

Analysis of Phenotypes Associated with a Hypomorphic Allele of *Rb* in the Mouse (*RbI3neo*)

Introduction

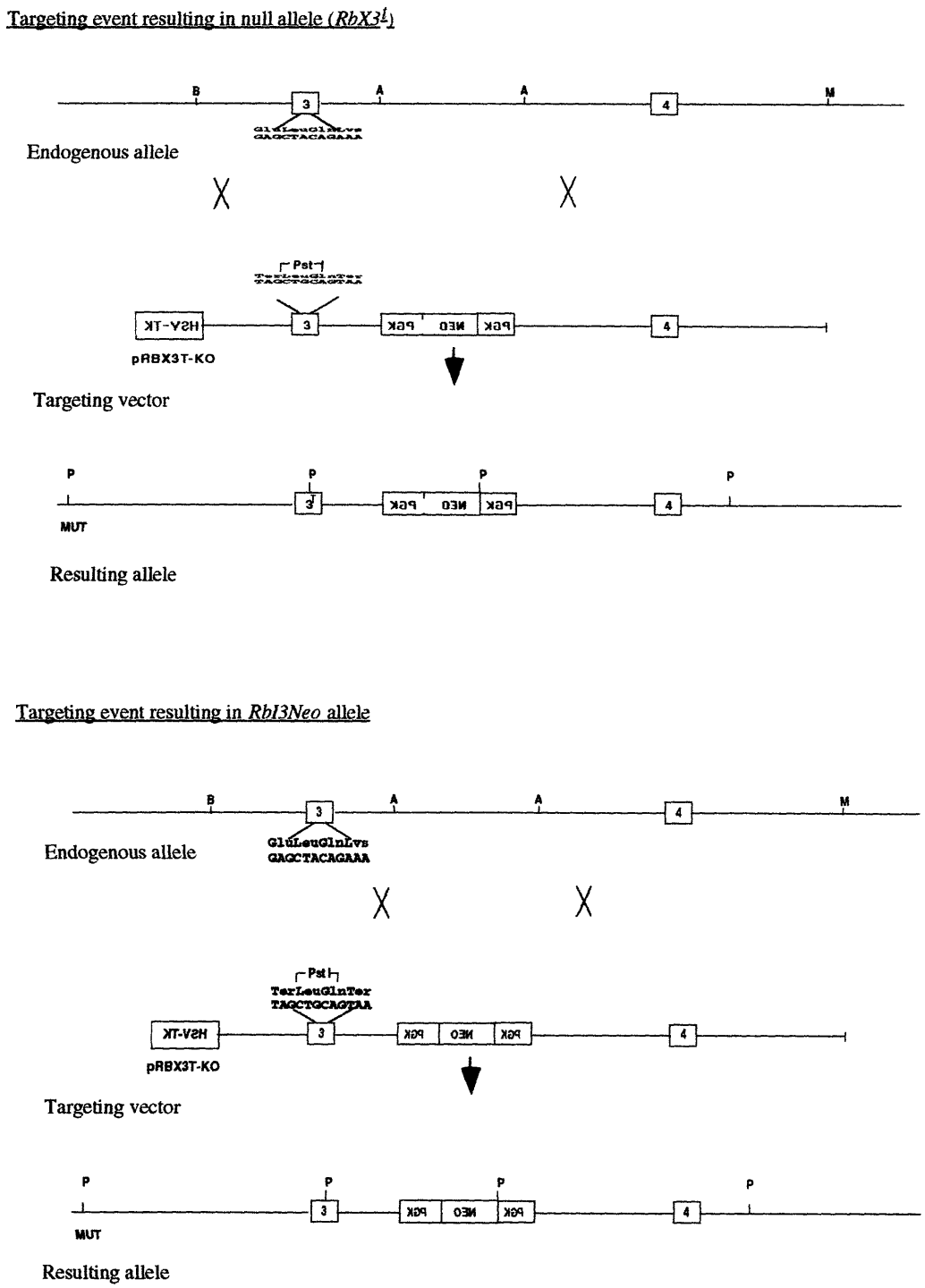
While the generation and analysis of the null alleles of *Rb* allowed significant insights into the role of *Rb* during murine development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), the mid-gestation embryonic lethality precluded examination of the role of *Rb* during later stages of development. One method used to overcome this problem, the generation of chimeric mice from *Rb*-deficient ES cells, has been discusses earlier in great detail (see Chapters 4 and 5). This appendix describes the generation and analysis of a strain of mice carrying an allele of *Rb* that causes significantly reduced levels of p*Rb* protein, but not complete elimination of full length protein as the allele described above does (Jacks et al, 1992). Animals heterozygous for this mutation develop intermediate lobe pituitary tumors histologically indistinguishable from those which arise in the null allele heterozygotes, albeit after a longer latency period. Almost all mice homozygous for this allele of *Rb* die within 6 hours of birth and this neonatal lethality is associated with a failure of the embryos to properly inflate their lungs. The underlying cause of this is not known. Thus, this allele has allowed identification of another window in development where *Rb* function is required for viability.

Materials and methods

Initial Generation of *RbI3Neo* +/- ES Cells

The initial clone of ES cells used to derive mice was created by Tyler Jacks with the same targeting vector as described previously (Jacks et al., 1992). A homologous recombination event induced by this targeting vector was designed to include two stop codons in the third exon of the *Rb* gene as well as place the neomycin resistance gene in the third intron to provide for positive selection of electroporated clones. In most cases the homologous targeting event occurred in such a way that both the stop codons and the *neo* gene were included. However, in one clone the homologous recombination occurred in a way that the neomycin resistance gene was inserted into the third intron of the *Rb* gene, but the stop codons were not introduced into the third exon (Figure D-1). ES cells containing this *Rb* allele was used to generate chimeras and will be referred to as the *RbI3neo* allele.

Figure D-1. Schematic of *Rbl3Neo* allele generation



Generation of a second independent *RbI3Neo* allele

Because all the *RbI3Neo* mice examined in the early stages of analysis were derived from a single ES cell clone, it remained possible that some observed phenotypes could be due to a second, linked and unknown mutation created in those ES cells. In order to address this possibility, a second allele of *RbI3Neo* was created by electroporation of D3 ES cells with a targeting vector including only the neomycin resistance gene in the third intron. Clones were electroporated and selected as previously described. Homologous recombinants were identified as previously described (Jacks et al., 1992). Analysis of 16 selected clones by Southern blot (as previously described) revealed 3 homologous recombinants. Two of these clones were used to create chimeric mice. One line of chimeric mice transmitted the *RbI3Neo* mutation through the germline. The phenotypes of these mice were indistinguishable from the initially derived allele, making it extremely likely that the phenotypes observed are the result of the mutation in the *Rb* allele and not some unknown, linked mutation induced during homologous recombination.

Generation of chimeric mice

Chimeric mice were generated by blastocyst injection as previously described (see Chapters 5 and 6).

Generation of strains

RbI3Neo mice carrying the mutation on two different genetic backgrounds were derived. The first background is a mixture of 129/Sv and C57/Bl6J derived from intercrosses of animals created by germline transmission by the chimera when mated to purebred C57/Bl6J females. The second background is purebred 129/SvJ derived from mating chimeras to purebred 129/SvJ females and identifying pups derived from germline transmission by PCR genotyping (Jacks et al., 1992).

Western blot analysis

Western blot analysis was performed as previously described (Jacks et al., 1992). Extracts were taken from fetal brain (12.5 days) as previously described (Jacks et al., 1992).

Southern blot analysis

Southern blot analysis was done as previously described (Jacks et al., 1992). A schematic representation of the products of the restriction digest and the location of the probe is shown in Figure D-2. A representative Southern blot is shown in Figure D-3.

Figure D-2. Schematic of genomic structure and expected restriction digest pattern using *Pst1* digested genomic DNA. *Pst1* sites are denoted by arrows. The probe used for the analysis is indicated with a black box.

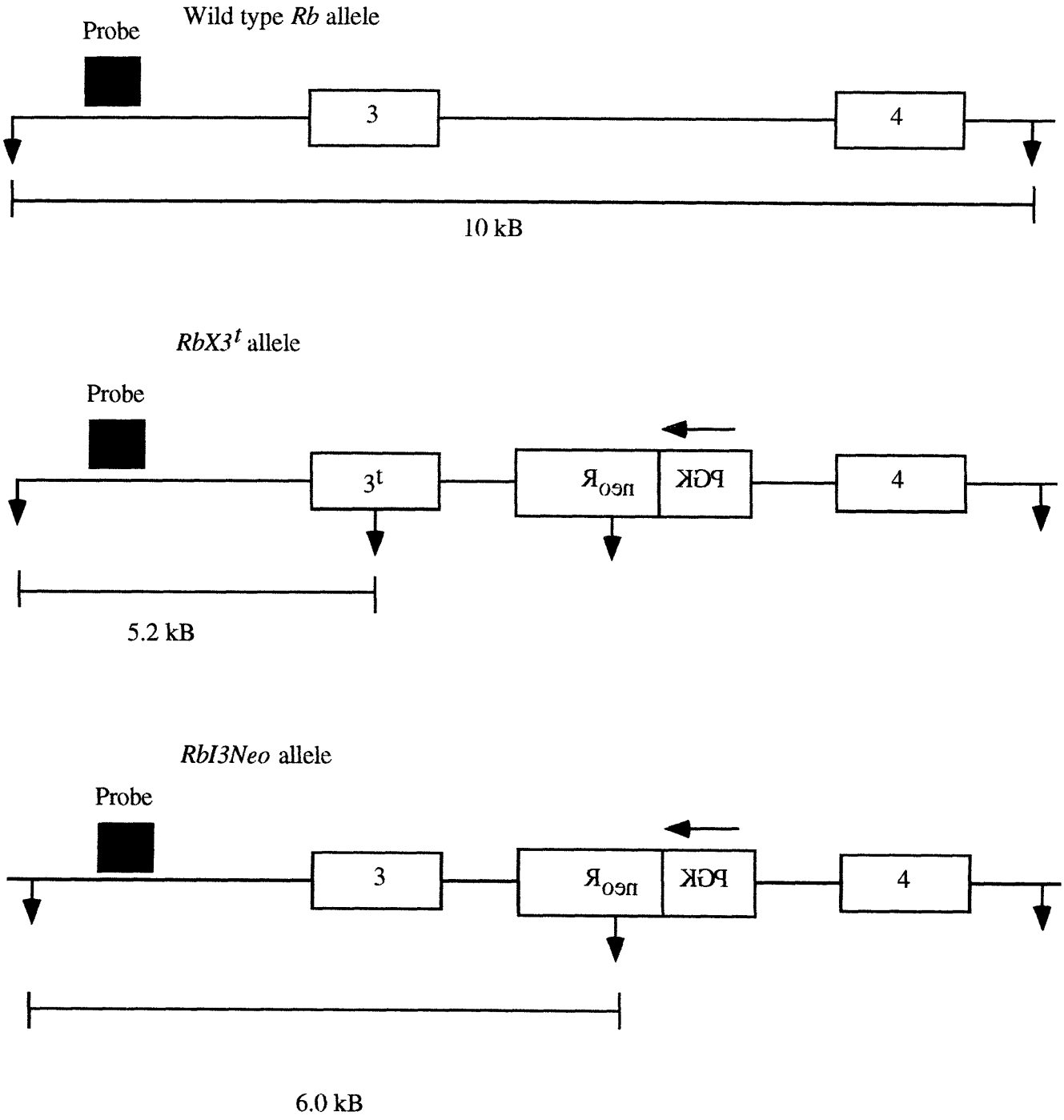
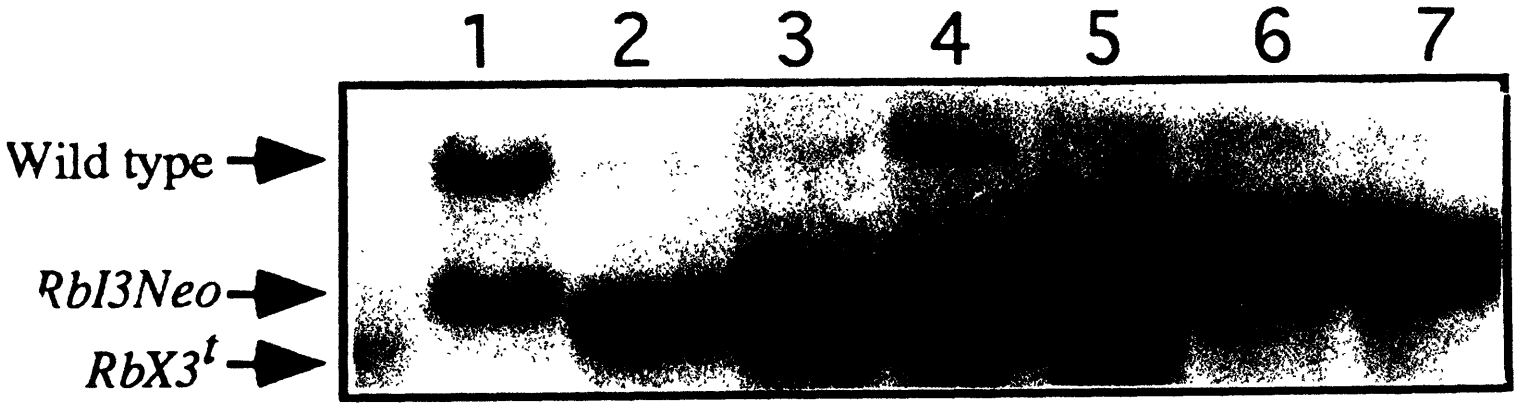


Figure D-3. Southern blot tissues and tumors from *RbX3^t* and *RbI3Neo* heterozygous animals. The positions of the wild type, *RbI3Neo*, and *RbX3^t* alleles are noted on the left. Lane 1, Tail DNA from an *RbI3Neo* heterozygote. Lanes 2-4, Pituitary tumor DNAs from RbI3Neo heterozygotes. Lanes 5-7, Pituitary tumor DNAs from *RbX3^t* heterozygotes. Note that in all cases the wild-type allele of *Rb* is reduced or absent from the pituitary tumor DNA.



RT-PCR

For RT-PCR, total RNA was isolated from brain, liver, and body wall (carcass) tissue of 13.5 day embryos via the RNAzol method (BiotecX Laboratories, Houston). Briefly, 13.5 embryonic brain, liver, and carcass were isolated and disaggregated in 100 microliters of PBS in an Eppendorf tube. 500 microliters of RNAzol B was added to this. 50 microliters of chloroform was added to this and the whole mixture placed on ice for 5 minutes. Then the tube was placed in an Eppendorf centrifuge and spun at 14,000 rpm for 15 minutes (at 4 degrees Celsius). The supernatant was then added to 300 microliters of isopropanol and then stored at -20 degrees Celsius for at least 12 hours. RNA was precipitated by centrifugation and then resuspended in 100 microliters of distilled water. Reverse transcription was performed in the following manner (recipes for solutions are included below).

Reverse transcription reaction
2 microliters 10X RT Buffer
2 microliters 10X dNTPs
2 microliters 1 microgram/microliter oligo-dT
1 microliter RNAsin
1 microliter Superscript (GIBCO-BRL)
10 microliters RNA solution

This reaction mix was placed at 37 degrees Celsius for one hour. Then 80 microliters of distilled water was added and the mixture boiled for 5 minutes. The mixture (cDNA solution) was then stored at -20 degrees Celsius until use. For PCR the following conditions were used. In this case, PCR primers were designed to amplify a 150 base pair fragment that would result from a splicing event from the 3' end of exon 2 into the neomycin resistance gene in the third intron (see Figures D-6 and D-7). PCR conditions were as follows (again recipes for the ingredients are included below).

Reaction Mix
19 microliters DNA mix
0.5 microliters oligo mix
0.5 microliters Taq polymerase

The following PCR program was used.

Step #	Temperature (Celsius)	Time (seconds)
1	94	60
2	94	36
3	55	36
4	72	180
Repeat steps 2-4 30 times		
5	72	554

PCR products were then visualized by running them out on a 2% agarose gel.

Recipes for RT-PCR solutions (all quantities are in microliters unless noted)

DNA mix
20 cDNA
48 10X PCR buffer
10 mM dNTPs
378 distilled water

Oligo mix
78 distilled water
6 *Rb*X3 (5'-AATTGCGGCCGCATCTGCATCTTTATCGC-3')
6 Neo 18.5 (5'-TCCTCGTGCTTTACGGTATC-3')

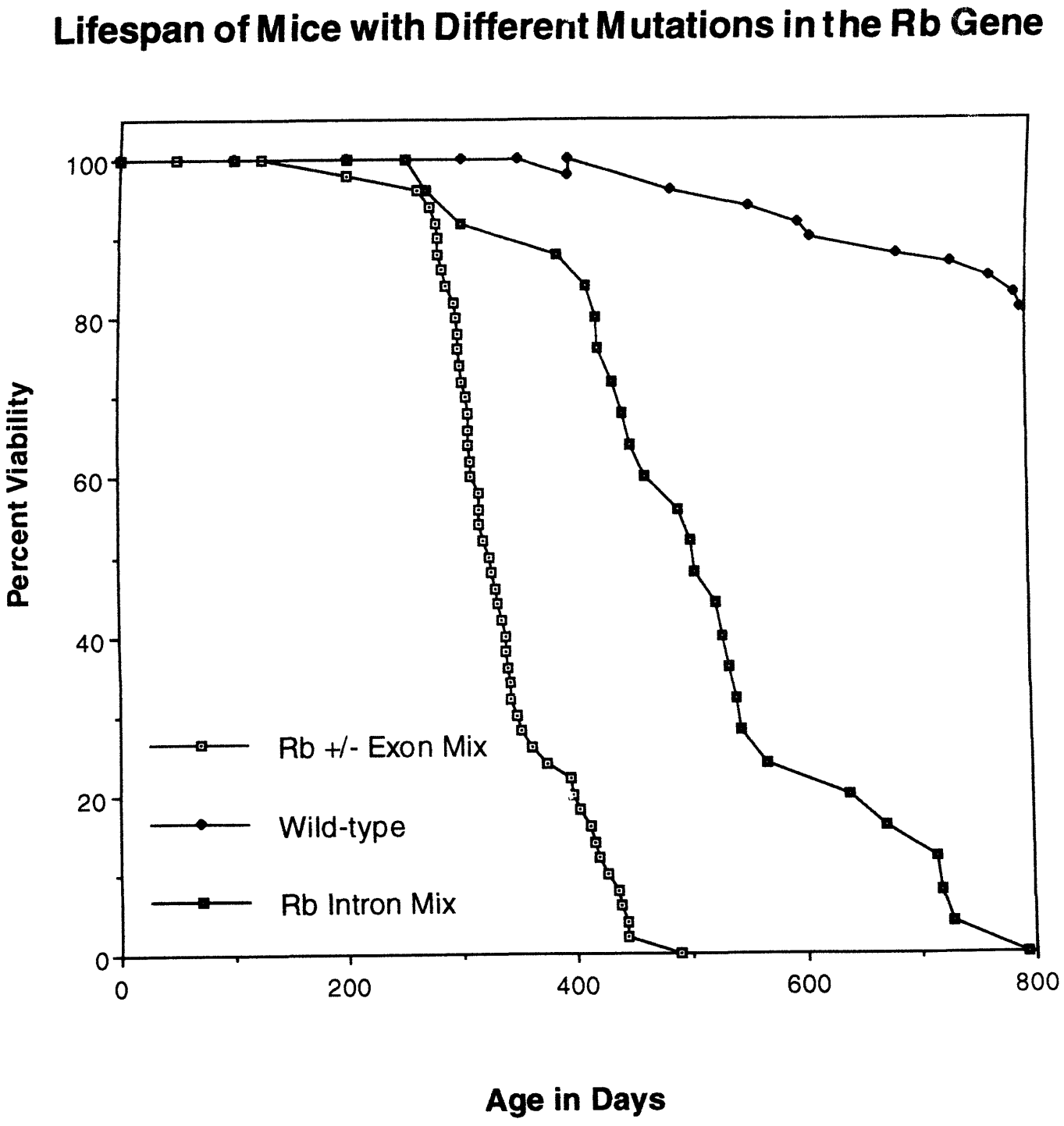
10X PCR buffer (final concentrations)
500 mM Tris-HCl (pH=8.3)
30 mM $MgCl_2$
750 mM KCl
100 mM DTT

Results

Heterozygous phenotype

Mice heterozygous for this allele of *Rb* were viable and fertile. They also developed the intermediate lobe pituitary tumors characteristic of *Rb* null heterozygotes (see Figure D-8). However, the latency of tumor development was significantly increased, both in a 129 and mixed genetic backgrounds (see Figure D-2). Southern blot analysis and in some cases PCR analysis revealed that, as in the other previously reported strains of mice, the wild-type allele of *Rb* was absent from the pituitary tumors (see Figure D-3).

Figure D-4. Survival of wild-type mice (50 mice) relative to RbI3Neo (25 mice) and RbX3^{t} (50 mice) heterozygotes. All animals are of a mixed genetic background.



Homozygous phenotype

While animals homozygous for the null allele of *Rb* die between days 13.5 and 14.5 of gestation with defects in fetal liver hematopoiesis and neurogenesis, mice homozygous for the *Rb* I3 allele are present at normal levels throughout gestation and survive until birth. However, the vast majority of homozygotes (and 100% of those derived on a purebred 129/Sv background) die during the first 4-6 hours after birth (see Table D1-3). These animals appear healthy when born, but quickly become cyanotic and appear to have difficulty breathing. Pathological analysis reveals that the mice never expand their alveolar spaces (data not shown). Despite extensive pathological analysis we have been unable to ascribe the neonatal lethality to any underlying cause. The only pathological abnormality seen consistently in these mice is the presence of bilateral cataracts (see Figure D-7). Rare surviving animals (all from a mixed background) are fertile and do not exhibit any significant histological abnormalities besides cataracts and pituitary tumors (Figure D-7). A summary of adult animals (defined as 3 weeks of age) derived from crosses of animals heterozygous for the *RbI3Neo* allele is shown below (Table D-2). In addition, results from a limited number of crosses of homozygous mutants by heterozygotes are shown on the following pages (Table D-3). Finally, litters from crosses between *RbX3*^{t} heterozygotes and *RbI3Neo* heterozygotes were examined just prior to birth. This analysis showed that RbI3Neo/RbX3^{t} compound heterozygotes also appear normal until birth (Table D-4).

Table D-1

Presence of normal *Rbl3Neo* homozygotes during development

(Derived from *Rbl3Neo* heterozygous intercrosses of 129 and mixed genetic background)

Developmental stage	Wild type	*Rbl3Neo +/-*	*Rbl3Neo -/-*
12.5 days p.c.	3	1	3
13.5 days p.c.	3	3	1
15.5 days p.c.	4	3	2
18.5 days p.c.	3	5	5
Immediately after birth (less than 2 hours)	4	7	2

Table D-2

Animals surviving to 3 weeks of age derived from *Rbl3Neo* heterozygote crosses

(data are derived from crosses of the two separately derived alleles)

Genetic Background	Wild-type	*Rbl3Neo +/-*	*Rbl3Neo -/-*
Purebred 129/Sv	44	72	0
Mix (129/Sv and C57/Bl6J)	54	96	5

Table D-3

Animals surviving to 3 weeks of age derived from *RbI3Neo* -/- by *RbI3Neo* +/- crosses.

Genetic Background	*RbI3Neo* +/-	*RbI3Neo* -/-
Mix (129/Sv and C57/Bl6J)	16	2

Table D-4

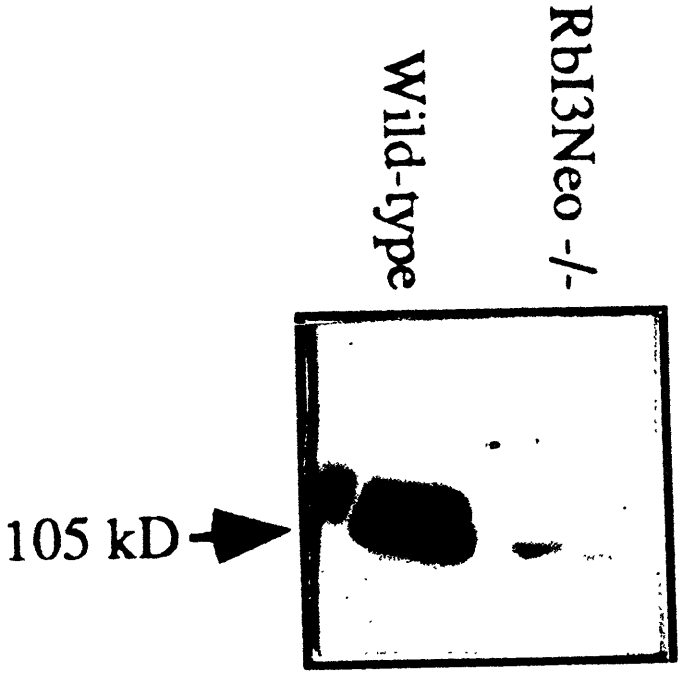
Results of crosses between *RbI3Neo* heterozygotes and *Rb*X3^t heterozygotes (From mixed genetic background). The number of normal embryos for each genotype is given. No abnormal embryos were observed.

Developmental stage	Wild type	Heterozygotes (of either allele)	*RbI3Neo/RbX3^t* homozygotes
18.5 days p.c.	3	5	5

Molecular characterization of the *Rbl3Neo* allele

Western blot analysis of lysates made from 12.5 day wild-type and mutant embryo brains revealed a substantial decrease in the amount of Rb protein in the homozygous *Rbl3Neo* embryos. 100 micrograms of protein was loaded for each lane. As can be seen in Figure D-5, the levels of pRb in the *Rbl3Neo* homozygous mutant animals are substantially reduced relative to a representative wild-type littermate. In addition, immunofluorescenct analysis of pRb levels in MEFs homozygous for the *Rbl3Neo* mutation confirmed a substantial reduction in pRb levels (data not shown).

Figure D-5. Western Blot analysis of pRb in intron mutant embryos. 100 micrograms of protein was loaded onto each lane. The migration of a molecular weight marker (105 kD) in another lane on the blot is noted.



RT-PCR

RT-PCR analysis of RNA extracted from 13.5 day heterozygous and wild-type embryos revealed an abnormal splice product present in all embryos carrying at least one copy of the *RbI3Neo* allele which was absent in all wild-type embryos. This product was consistent with one derived from splicing into a strong, consensus splice acceptor sequence revealed when the neomycin resistance gene is included in the opposite transcriptional orientation to the gene being disrupted (Figure D6-7 and Jacks et al., 1994). This abnormally splice product would be predicted to give rise to a truncated protein and likely explains the reduction of pRb levels in extracts from homozygous mutant *RbI3Neo* embryos.

Figure D-6

Products of the RT-PCR described above were resolved on 2% agarose-TAE gels. (Lane 1, 123 bp ladder; Lane 2, wild-type brain; Lane 3, wild-type liver; Lane 4, wild-type carcass; Lane 5, *RbI3Neo* +/- brain; Lane 6, *RbI3Neo* +/- liver; Lane 7, *RbI3Neo* +/- carcass. The presence of a non-specifically amplifying band is noted (n). The presence of the band (~150 bp) predicted to result from the putative abnormal splicing event is noted (s).

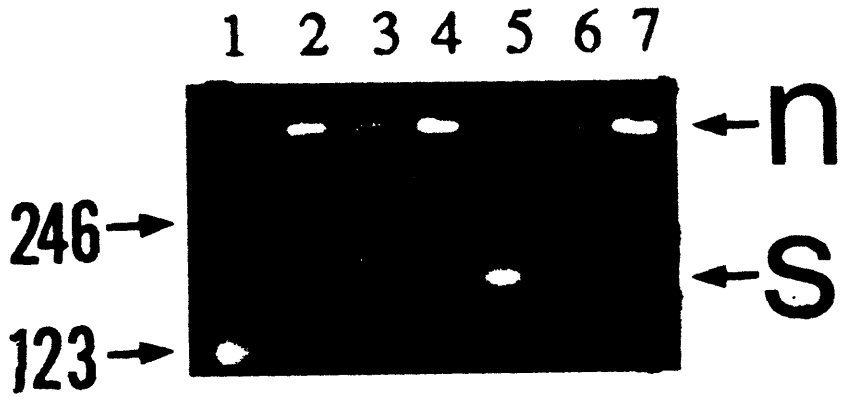
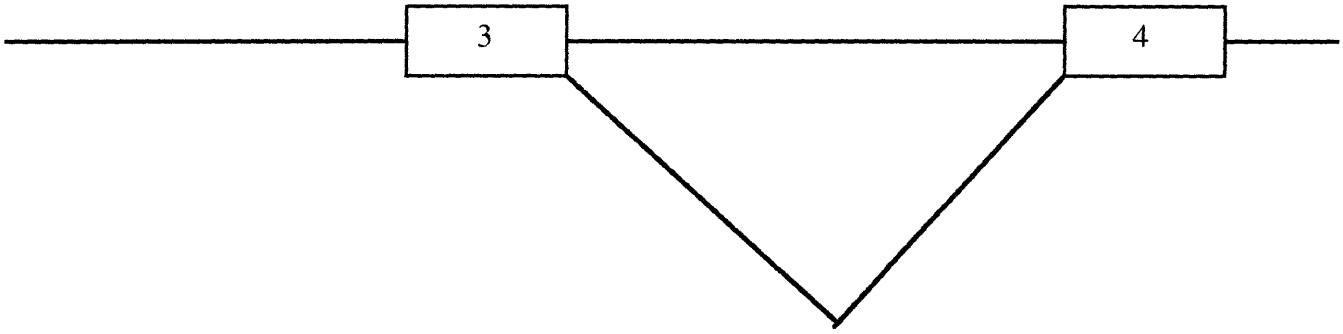


Figure D-7. Schematic diagram of the normal splicing event and the abnormal event taking place at some frequency from the *RbI3Neo* allele.

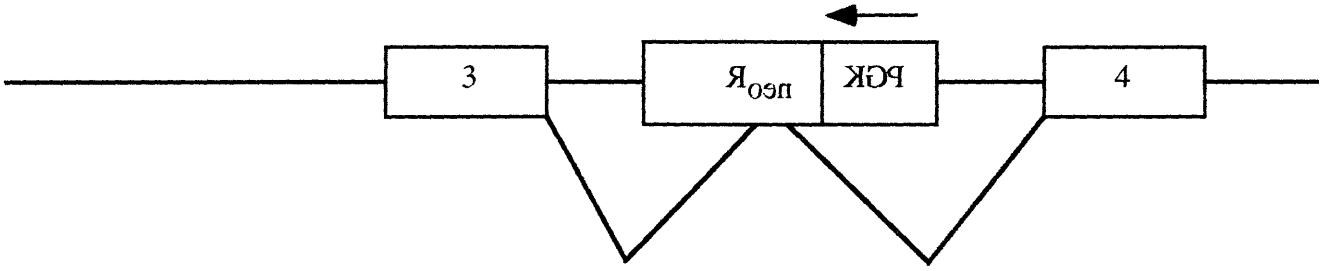
Wild type *Rb* allele

(Normal splicing pattern)



RbI3Neo allele

(Abnormal splicing pattern)



Discussion

The data in this appendix show that while complete loss of *Rb* function results in embryonic lethality at approximately 13.5-15.5 days, a reduction in the amount of p*Rb* to about 10% of wild-type levels causes neonatal lethality. This reduced amount is sufficient for embryos to survive past 15.5 days of gestation and no abnormalities in fetal hematopoiesis or in neurogenesis have been observed histologically. In fact, when compound heterozygotes are produced by mating *RbI3Neo*+/- animals to *Rb* null heterozygotes, the same neonatal lethality is observed, suggesting that even smaller amounts of *Rb* protein are sufficient to allow sufficient fetal hematopoiesis and neurogenesis.

The genomic structure of this allele has broader implications in the field of gene targeting. The presence of the putative splice acceptor and splice donor sites within the neomycin resistance gene when it is in reverse orientation to the targeted gene was first noted during the analysis of the *Nf-1* gene knockout(Jacks et al., 1994). In the case described here, the abnormal splicing event would be predicted to create a severely truncated non-functional protein product. Interestingly, this abnormal splicing event does not occur with 100% efficiency since *RbI3Neo* homozygotes still produce a small amount of full length protein.

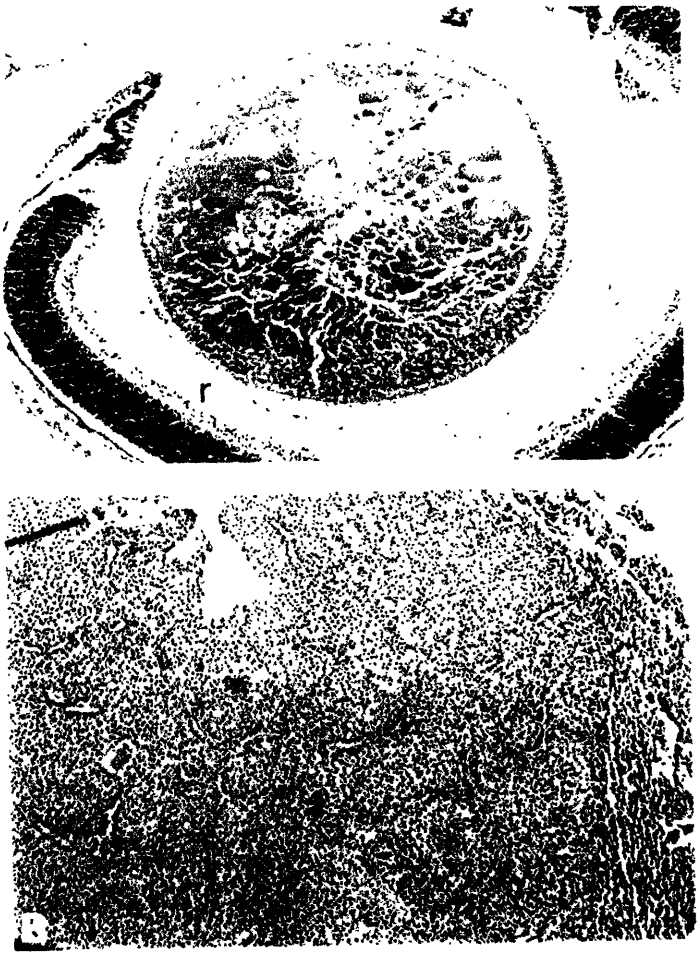
The value of this mouse strain will be expanded greatly if the underlying cause of the neonatal lethality can be understood. Despite extensive histological analysis, an underlying cause has yet to be determined. If and when the cause of lethality is determined, this mouse will identify another system which is dependent on the presence of normal amounts of pRb for normal function.

Figure D-8

Pathology associated with the *Rbl3Neo* mutation.

(A) Section through eye of newborn *Rbl3Neo* homozygote (100X). The presence of a cataract (c) in the lens capsule as well as a normal appearing retina (r) are indicated

(B) Section through intermediate lobe pituitary tumor (100X) from a *Rbl3Neo* heterozygote.



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APPENDIX E

Generation of ES Cells Deficient for Multiple Members of the *Rb* Gene Family

In order to further advance the utility of the chimeric mouse system to study *Rb*-function, I initiated efforts to derive ES cells carrying deficiencies in different combinations of the *Rb*-family members. Because any mouse (unless somehow dramatically rescued by some suppressor mutation) carrying a germline homozygous deficiency in the *Rb* gene will die during mid to late gestation, these ES cells will prove a valuable reagent to probe the potential overlapping functions of these proteins in adult tissues. In addition, the neonatal synthetic lethality seen in animals deficient for both *p107* and *p130* (Cobrinik et al., 1996) further reinforces the need for such reagents.

In this appendix I describe my efforts to develop such systems and comment on how these may be used in the future. I have succeeded in isolating cell lines which are deficient for *p107* and *p130* as well as cell lines which are deficient for both *Rb* and *p107*. George Mulligan, a postdoctoral associate in the laboratory, has isolated cells which are deficient in both *Rb* and *p130*.

Materials and Methods

All procedures for ES cell generation, *ROSA-26* genotype determination, *Rb*-genotype determination, and ES cell culture and injection in general are described in Chapter 5. Analysis of *p107* genotype status was performed as described in Appendix C. All histological examinations were performed as previously described.

p130 PCR genotype determination

p130 genotype was determined by a PCR-based assay (Cobrinik et al., 1996). Genomic DNA was isolated as described above and amplified in the following conditions.

Results

Generation of ES Cells

The chart below summarizes the generation of ES cells with the desired genotype.

Cross	# Females	# Blastocysts	# ES cell lines
p107+/-;p130-/- intercrosses	10	36	11
Rb+/-;p107-/-;ROSA-26 male X *Rb+/-;p107+/-* female	3	7	2

From the *p107/p130* crosses one *p107-/-;p130-/-* ES cell line was established. *Zfy* PCR (Page et al., 1987; Sah et al., 1995) failed to show a PCR product indicating the line was female. From the *Rb/p107* crosses one *Rb-/-;p107-/-*;ROSA ES cell line was established. *Zfy* PCR indicated that it was male.

Generation of Chimeras from *p130-/-; p107-/-* ES cells

Two rounds of injection were performed with these cells resulting in the generation of 5 moderate to highly chimeric animals. Of these five animals (all born in November 1995) two have died prior to four months of age. One of these animals exhibited the presence of tingible body macrophages throughout the white pulp of the liver (see Figure E-1) and also leakage of proteinasceous material into the glomerular space of the kidney (data not shown). No other abnormalities (including bone formation) were detected (see Figure E-1). Histological analysis of the second animal revealed the presence of a severe stomach ulcer as well as a thrombus of blood

lodged in the heart, which probably caused the death of the animal (data not shown). Again, no abnormalities in the formation of bone were observed.

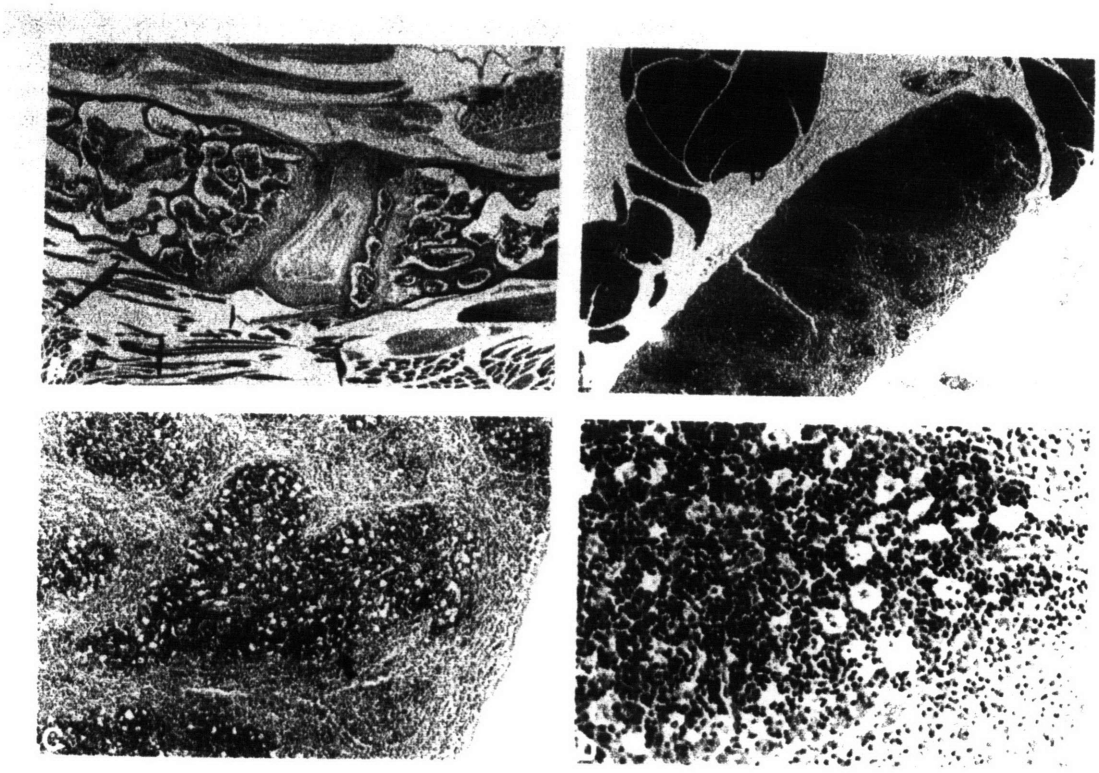
Figure E-1

p130/p107 Chimera Histology (see following page)

Hematoxylin and eosin stained sections from a 3 month old chimera derived from p107-/-;p130-/- ES cells.

A. Section through femur showing normal bone formation (40X). Despite the fact that *p107-/-;p130-/-* neonates show severely shortened limbs associated with bone deposition defects, all chimeras examined so far have normal limbs, and two chimeras (over 80% chimeric as judged by coat color) have been histologically examined with no noted defects in bone formation.

B-D. Sections through spleen 40X (B), 100X (C), 400X (D). Normal pancreas (p) and abnormal spleen (s) are shown. Note the presence of abnormal white pulp (arrow in C) in the spleen.



Injection of *Rb-/-;p107-/-;ROSA-26* ES cells

Three rounds of injection were performed with these cells. To date, no viable chimeric offspring have been recovered, although three potential chimeric pups were born only to be cannibalized by the birth mother.

Discussion

While the analysis of chimeras generated from these ES cells is in very preliminary stages, the analysis of chimeras derived from the *p130/p107* doubly deficient ES cells has already revealed pathological abnormalities. Experiments following up these observations will no doubt be valuable in increasing our understanding of these genes' functions. Interestingly, the preliminary analysis suggests that the bone defect seen in neonates with a germline genotype of *p107-/-;p130-/-* may be a non-cell autonomous defect as the four very strong chimeras created to date have normal limbs and have no noticeable histological defects.

In addition to the cell lines described here, George Mulligan, a post-doctoral associate in our laboratory, has successfully isolated *Rb-/-;p130-/- ROSA-26* ES cells. He is now using the cells he isolated as well as the cells described above to try to generate ES cells deficient in all three *Rb*-family genes via methods similar to those described in Chapters 5 and 6. If and when these *Rb-/-;p130-/-;p107-/-* ES cells are created, they will useful in addressing the role of *Rb*-family functions in a variety of systems including chimera generation and *in vitro* ES generation (Robertson, 1987).

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