Targeted disruption of *p107*: functional overlap between *p107* and *Rb*

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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 with *Rb*^{+/-} mice but developed multiple dysplastic lesions of the retina. Embryos homozygous for both *Rb* and *p107* died at ~11.5 days of gestation, 2 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.

[Key Words: *p107*; *Rb*; gene targeting; apoptosis; retinal dysplasia; liver]

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The retinoblastoma gene (*RB*) is a prototype of the tumor suppressor genes. In humans, mutation of *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, 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, which targets the pRB family of proteins as well as p53 [Windle et al. 1990; Al-Ubaidi et al. 1992], or human papillomavirus type 16 [HPV-16] E7 [which inactivates the pRB family] in a p53-mutant genetic background [Howes et al. 1994]. 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 the G₁ 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

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vivo and to repress E2F-dependent transcription when overexpressed. The coexpression 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 (Beijersbergen et al. 1994a; Ginsberg et al. 1994), whereas pRB interacts with E2F-1, E2F-2, and E2F-3 (Lees et al. 1993) and p130 interacts 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 G₀, whereas the pRB/E2F and p107/E2F complexes appear later in G₁ 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 a 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 Figure 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 (PGK) promoter, in the antisense 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 blot 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 that transmitted the *p107* mutation through the germ line. 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 blot 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) that 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 ~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).

Figure 1. Targeted disruption of the mouse *p107* gene. **(A)** The restriction map of a targeting vector and wild-type and mutant *p107* alleles. Homologous recombination would result in the replacement of a 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 cassettes, which are in the opposite direction to *p107*. (■) *p107* exon sequence containing the first ATG translation initiation sequence. A *p107* gene 5'-flanking probe and a 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 *HpaI* and *SphI* for the wild-type and mutant alleles. (R) *EcoRI*; (P) *PstI*; (H) *HindIII*; (Hp) *HpaI*; (Sp) *SphI*. **(B)** Southern blot analysis of *HpaI* plus *SphI*-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 *HpaI* and *SphI* and probed with the 5' probe. The genotypes are depicted at the top.

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 was also normal compared with 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 (Cobrinik et al., this issue).

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 G₁/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 4–6) derived from 12.5-day-old 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

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*^{-/-} mouse embryo fibroblasts using a monoclonal antibody against 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 mass standards are indicated at left. The position of p107 and of cross-reactive IgG are 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 mass markers are indicated at left. (Bottom) 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 (top) and p130 (bottom). Note that pRB and p130 levels are not altered significantly in *p107* mutant embryos.

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 ~1 year of age. PCR analysis of seven pituitary tumors from these mice indicated that the wild-type allele of *Rb* was absent, whereas the wild-type allele of *p107* was retained (data not shown). Thus, there appears to be no selective advantage to the 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 by intercrossing *Rb*^{+/-}; *p107*^{+/-} mice or crossing these animals 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,B). Litters contained pups of approximately uniform size at birth, but after several days, some runt pups were evident. Genotyping showed that the runt pups were consistently *Rb*^{+/-}; *p107*^{-/-}, and they were born at somewhat lower than the expected frequency. Of 81 pups genotyped at 1 week of age from *Rb*^{+/-}; *p107*^{+/-} by *p107*^{-/-} crosses, 15 (19%) were *Rb*^{+/-}; *p107*^{-/-}, which is significantly fewer than the expected frequency of 25%. All of these mice were severely growth retarded for several weeks, averaging ~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 grams (*n* = 8), whereas that of *Rb*^{+/-}; *p107*^{+/-} mice was 6.2 ± 0.3 grams (*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 grams (*n* = 10) for *Rb*^{+/-}; *p107*^{-/-} compared with 11.0 ± 0.6 grams (*n* = 8) for *Rb*^{+/-}; *p107*^{+/-}. Approximately 25% of these *Rb*^{+/-}; *p107*^{-/-} mice survived beyond 3 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. We have been unable to determine the basis of the reduced body size or viability of the *Rb*^{+/-}; *p107*^{-/-} animals. The majority of the surviving male *Rb*^{+/-}; *p107*^{-/-} animals were fertile (Table 1C,D) and otherwise normal up to 12 months of age. However, ~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 and 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 up to 1 year of age, there is no

Table 1. Offspring viability of $Rb^{+/-};p107^{+/-}$ mice at 3–4 weeks of age

A. $Rb^{+/-};p107^{-/-} \times Rb^{-/-};p107^{+/-}$						
Genotype ^a <i>Rb</i>	+/+	+/+	+/+	+/-	+/-	+/-
<i>p107</i>	+/+	+/-	-/-	+/+	+/-	-/-
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. $Rb^{+/-};p107^{-/-} \times p107^{+/-}$						
Genotype ^a <i>Rb</i>	+/+	+/+	+/-	+/-		
<i>p107</i>	+/-	-/-	+/-	-/-		
Expected ratio	1	1	1	1		
Numbers obtained	148 (1.0)	179 (1.2)	157 (1.1)	38 (0.26)		
C. $Rb^{+/-};p107^{-/-}$ male $\times$ $p107^{+/-}$ female						
Genotype ^a <i>Rb</i>	+/+	+/-				
<i>p107</i>	-/-	-/-				
Expected ratio	1	1				
Numbers obtained	69 (1.0)	14 (0.2)				
D. $Rb^{+/-};p107^{-/-}$ male $\times$ $Rb^{+/-};p107^{-/-}$ female						
Genotype ^a <i>Rb</i>	+/+	+/+	+/-	+/-		
<i>p107</i>	+/-	-/-	+/-	-/-		
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-week-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).

^aSeveral genotypes of mice that are embryonic lethal because of an $Rb^{-/-}$ genotype were excluded.

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 (Maandag et al. 1994; Williams et al. 1994), this tumor is observed upon retinal expression of viral oncogenes that bind and inactivate all three members of the pRB family in animals with compromised p53 function. Therefore, we examined carefully 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 of the 12 were affected in both eyes. Typically, each eye exhibited five to seven individual lesions consisting of small white bodies, usually in clusters (Fig. 4A). No retinal abnormalities were detected in any of 8 $p107^{-/-}$ mice examined or in >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 (Fig. 4B–D). 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 (Fig. 4E,F), which could represent precursors to retinal dysplasia.

Early death of $Rb^{+/-};p107^{-/-}$ embryos

pRB is required for normal mouse development (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). $Rb^{-/-}$ embryos die between 13.5 and 15.5 days of gestation, with excessive apoptotic cell death and inefficient erythroid differentiation. 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 (Clarke et al. 1992; Jacks et al. 1992; Lee 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). TUNEL stain-

Figure 3. Phenotypes of $Rb^{+/-};p107^{-/-}$ mice. (A) Growth retardation of $Rb^{+/-};p107^{-/-}$ mice. Two-week-old littermates from an $Rb^{+/-};p107^{+/-}$ by $p107^{-/-}$ cross are shown. The animal at left is $Rb^{+/-};p107^{-/-}$ and is significantly growth retarded compared with 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 an $Rb^{+/-};p107^{-/-}$ by $Rb^{+/-};p107^{+/-}$ cross. (C) Vaginal atresia in $Rb^{+/-};p107^{-/-}$ females. Note that the abdomen and perineum are distended owing to fluid accumulation in the uterus.

ing of histological sections from the CNS and liver of this embryo confirmed that the death occurred by apoptosis (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 with $Rb^{+/-};p107^{-/-}$ 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 the induction of DNA synthesis in the nuclei of differentiated myotubes (Endo 1992). Although aberrant cell cycle reentry 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 paper; Corbin et al., this issue), 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 de-

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 caused by 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 C. (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. Note that the detached sclera (B,E,F) and ganglion cell layer (E,F) are artifacts of the histological processing and sectioning. Magnifications (B,C,E) 100 $\times$; (D,F) 400 $\times$.

gree 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 >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 (e.g., see Cobrinik et al. 1993; Schwarz et al. 1993; Zhu et al. 1993; Beijersbergen et al. 1994a,b; 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 show 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 CNS compared with *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 (Cobrinik et al., this issue); 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 mutant mice might be caused by 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 mu-

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 out of 22) of *Rb*^(-/-) embryos were recovered alive at E12.5.

tants 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.

Because *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 depletion 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 suggest 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 caused by photoreceptor cell death in transgenic mice following the expression of the HPV-16 E7 oncogene exclusively in photoreceptor cells and the 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 (S. Mukai and T. Jacks, unpubl.), suggesting that degeneration of the retinal layer in the E7 transgenic mice 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.

These observations suggest that even subtle differences in the genetic control of cell growth and differentiation in equivalent cell types in mice and humans can affect the particular tumor phenotype caused by germline mutations in the two species. For example, it is possible that in the developing retina in humans, *RB* is the sole member of the gene family responsible for normal growth control, perhaps because *p107* is simply not expressed or not sufficiently active. As such, *RB* mutation would be sufficient for the development of retinoblastoma in this species. Perhaps owing to differences in the expression pattern of the *Rb* family members in mice, both *Rb* and *p107* may be capable of providing essential growth control functions in the relevant cells in the developing retina, such that both genes must be mutated to induce abnormal proliferation. On the basis of the current findings as well as those of Howes et al. (1994), we believe that the loss of *Rb* and *p107* function is still not sufficient for the development of retinoblastoma in the mouse and that *p53* mutation and consequent escape from apoptosis is another critical event in tumorigenesis in this tissue. This possibility can be tested directly by creating animals mutant for *Rb*, *p107*, and *p53*. Interestingly, the apparent requirement for *p53* inactivation is another distinct feature of retinoblastoma in mice, as *p53* mutations have not been reported in human retinoblastoma.

Taken together, the data presented here are consistent with the notion that p107 and pRB can serve related functions in growth control and/or differentiation in some developmental contexts and in a limited fashion in the inhibition of tumor formation. Because p107 and pRB both interact with E2F but associate with different subsets of E2F polypeptides (Beijersbergen et al. 1994a; 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

Figure 6. Histopathology of $Rb^{-/-}; p107^{-/-}$ embryos. (A,B) Sections of spinal cord from $Rb^{-/-}$ (A) and $Rb^{-/-}; p107^{-/-}$ (B) embryos at E11.5. Note that compared with 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,D) Liver sections from $Rb^{-/-}$ (C) and $Rb^{-/-}; p107^{-/-}$ (D) embryos at E12.5. The $Rb^{-/-}; p107^{-/-}$ section shows areas of acellularity and numerous pyknotic nuclei, whereas the $Rb^{-/-}$ liver is normal at this stage of development. (E,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, 100 $\times$.

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 $\times$ SSC, 0.1% SDS at 65 $^{\circ}$ C). Several clones were isolated, and a λ 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 mapped extensively 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 *Pst*I mouse genomic fragment and 3.0-kb *Hind*III–*Not*I (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 *Not*I site in the construct was used to linearize the plasmid before electroporation into ES cells.

ES cell culture, transfection, and selection

The D3 ES cell line derived from strain 129/Sv, was grown on feeder layers of γ -irradiated (1500 rads for 30 min) embryonic fibroblasts in Dulbecco's modified Eagle medium supplemented with 15% heat-inactivated fetal calf serum (Hyclone), 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 1000

U/ml of mouse leukemia inhibitory factor (ESGRO; GIBCO BRL). Cells (8×10^6) were electroporated in 0.8 ml of medium with 40 μ g of linearized targeting vector DNA at 400 V/250 μ F in a Bio-Rad Gene Pulser and were plated on feeder cells. Selection with 300 μ g/ml of G418 (GIBCO BRL) and 2 μ M gancyclovir was started 48 hr later. Three hundred doubly resistant colonies were selected for screening by Southern blot 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 of proteinase K overnight at 37 $^{\circ}$ C. Samples were extracted with phenol-chloroform, and genomic DNA was isolated by ethanol precipitation. Genomic DNA (10 μ g) from each clone was digested with *Hpa*I and *Sph*I, 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 $^{\circ}$ 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 μ g/ml of salmon sperm DNA at 65 $^{\circ}$ C. The 5' hybridization probe was a 950-bp *Rsa*I fragment derived from a 1.5-kb *Hpa*I–*Eco*RI fragment of *p107* genomic DNA, which contained sequences not included in the targeting vector. A 500-bp *Hae*III fragment derived from a 1-kb *Eco*RI–*Sph*I 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 $^{\circ}$ 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 owing 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- μ l reactions in buffer containing 60 mM Tris-HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 1 mM each dNTP, 1 unit of *Taq* polymerase (Promega), and 1 μ M 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 Sacchi 1987] and enriched for poly(A)⁺ RNA using oligo(dT)-cellulose. Samples (4 μ g) 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 at pH 7.0, 250 mM NaCl, 0.1% P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml of aprotinin, 1 μ g/ml of leupeptin, 0.5 mM DTT, and 0.5 mM sodium fluoride) and centrifuged for 10,000g 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 hr. 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, Canji) or polyclonal anti-p130 antibody (C-20, Santa Cruz) and then subsequently incubated with a goat antirabbit or antimouse 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,

Massachusetts Eye and Ear Infirmary Formulary, diluted 1:1 with sterile BSS) to the cornea of each eye. The pupils dilated fully within 5 min. 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, in prep.).

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 μ m, and stained with haematoxylin and eosin. For immunohistochemistry, embryo sections were deparaffinized and rehydrated before a 20-min incubation in block solution: 2% goat serum, 1% horse serum, 0.1% Triton X-100 in PBS. Sections were then incubated for 1 hr with antisarcomeric actin monoclonal antibody (Sigma) diluted 1:1000 in block. After three 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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