

Rb and p107 are required for normal cerebellar development and granule cell survival but not for Purkinje cell persistence

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SUMMARY

The involvement of the retinoblastoma gene product (Rb) and its family members (p107 and p130) in cell cycle exit and terminal differentiation of neural precursor cells has been demonstrated *in vitro*. To investigate the roles of Rb and p107 in growth, differentiation and apoptosis in the developing and mature cerebellum, we selectively inactivated either Rb alone or in combination with p107 in cerebellar precursor cells or in Purkinje cells. In our mouse models, we show that (1) Rb is required for differentiation, cell cycle exit and survival of granule cell precursors; (2)

p107 can not fully compensate for the loss of Rb function in granule cells; (3) Rb and p107 are not required for differentiation and survival of Purkinje cells during embryonic and early postnatal development; (4) Rb function in Purkinje cells is cell autonomous; and (5) loss of Rb deficient CNS precursor cells is mediated by p53-independent apoptosis.

Key words: Cre-LoxP system, Cerebellar development, Rb, Engrailed-2, p107, Granule cell, Purkinje cell, Mouse

INTRODUCTION

The retinoblastoma (Rb) gene family encodes a group of related proteins that participate in several aspects of cell growth and differentiation, including cell cycle regulation and control of gene expression. Rb, p107 (Rb1 – Mouse Genome Informatics) and p130 (Rb2 – Mouse Genome Informatics) most closely resemble each other in the so-called pocket region, which is composed of two domains: the A and B boxes. Although Rb has little similarities to the other family members outside the pocket domain, p107 and p130 are more closely related to each other. The highly conserved pocket region is crucial for binding and regulation of many cellular proteins, among them the E2f family of transcription factors.

E2fs comprise a group of at least six closely related proteins that regulate the expression of genes involved in cell cycle progression, differentiation, development and apoptosis. Cellular and biochemical analyses as well as studies of mutant mouse strains derived from gene targeting indicate distinct *in vivo* functions of Rb, p107 and p130. Although Rb and the upstream components of its pathway are mutated in many human cancers (reviewed by Weinberg, 1995), mutations in p107 have not been observed and mutations in p130 have been identified so far only in small cell lung carcinomas (Claudio et al., 2000; Helin et al., 1997). However, in mice only Rb loss has been directly associated with tumorigenesis, whereas loss of the other pocket proteins contributed only to tumorigenesis in combination with loss of Rb. In accordance with this notion,

mice that lack one Rb allele in a *p107*^{-/-} background do not show enhanced tumorigenesis.

During development, Rb is expressed from E9.5 onwards in both, mitotic and non-mitotic compartments of the brain folds of the neural tube (Jiang et al., 1997). In the adult brain, Rb is ubiquitously expressed, including cerebellar granule and Purkinje cells (Utomo et al., 1999). Rb knockout mice show embryonic lethality by E13–E15 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992) owing to major defects in hematopoiesis and central nervous system development. Major findings in the Rb-deficient CNS are increased and ectopic proliferation, and massive apoptosis, whereas Rb-deficient neurons in chimeric mice survive and differentiate, although with an abnormally high proportion of cells arresting in the G2 phase of the cell cycle (Lipinski et al., 2001). In contrast to the latter findings, Rb-deficient neuronal precursor cells in telencephalon-specific Rb mutants (Ferguson et al., 2002) showed ectopic cell divisions but not widespread apoptosis. Although these neuronal precursors differentiated, their fate in the adult CNS cannot be further investigated due to early postnatal lethality of the mice.

In contrast to the detrimental effects of Rb inactivation, mice lacking *p107* or *p130* develop normally and do not exhibit phenotypic aberrations (Cobrinik et al., 1996; Lee et al., 1996). Functional overlap within this gene family is suggested by ossification defects in *p107/p130* knockout mice and by retinal dysplasia in *Rb*^{+/-}; *p107*^{-/-} mice.

We studied the roles of Rb and p107 in cerebellar development

by conditional inactivation of Rb either in all precursor cells of the cerebellar vermis [line *En2cre*-22 (Zinyk et al., 1998)] or selectively in Purkinje cells [L7-cre (Marino et al., 2002)] and complemented the experiments by introducing p107 null alleles. We chose the cerebellum to study the roles of Rb and p107 in development, differentiation and cell death, as it consists of a limited number of distinct cell types, and because its development and architectural organization are extremely well documented. The use of a Cre transgenic lines with an expression limited to the cerebellar vermis or to Purkinje cells allows to study even severe effects on growth or cell loss without being lethal.

MATERIALS AND METHODS

Generation and screening of compound mutant mice

We crossed *En2cre* mice (line Tg22) with *Rb^{LoxP/LoxP}* or *Rb^{LoxP/LoxP}; p107^{-/-}* mice in order to obtain compound mutants as shown in Table 1. *p53^{-/-}* (*Trp53^{-/-}* – Mouse Genome Informatics) mice were used for the rescue experiment. Genotyping was performed according to published protocols (Marino et al., 2000; Robanus-Maandag et al., 1998).

Cell separation

The cerebellar vermis was dissected from two postnatal day 8 *En2cre;Rb^{LoxP/LoxP}* and from two *Rb^{LoxP/LoxP}* in cold Ca²⁺- and Mg²⁺-free PBS (PBS-CMF), meninges and choroid plexus were carefully removed. The Percoll gradient separation was performed according to Hatten (Hatten, 1985). Approximately 40,000 cells from each fraction were centrifuged on serial glass slides using a Cytospin machine. Cytospins were dried at room temperature and stored at -20°C. Genomic DNA was extracted directly from the cell suspension according to standard protocols.

PCR analysis of recombination

PCR analysis of Cre-mediated recombination on the cerebellar cell fractions was performed on genomic DNA using Rb212, Rb18 and Rb19E primers, yielding a 283 bp product for the unrecombined *Rb^{LoxP}* allele and a 260 bp product for the recombined RbΔ19 allele. For details see Marino et al. (Marino et al., 2000).

Analysis of proliferation and apoptosis

In order to examine the fraction of cells in the cerebellar EGL and IGL that are actively proliferating, P15 littermates were intraperitoneally injected with 50 mg/kg body weight with 5'-bromo-2'-deoxyuridine (BrdU) and killed 4 hours after injection. BrdU was immunohistochemically detected on sections of formalin fixed and paraffin wax-embedded brains (see Histological analysis). Apoptotic cells were detected with the TUNEL assay kit (Roche).

BrdU-positive nuclei and the total number of nuclei were counted in five high power fields separately in EGL and IGL. We counted corresponding areas located in the dorsal vermis (lobule VI and VII) where the phenotypic abnormalities were most prominent owing to the transgenic expression pattern. As the EGL of a wild-type mouse is thinner than the EGL of the mutant (and vice versa for the IGL), we decided to determine the labeling index by counting the number of positive cells and all the cells belonging to the EGL or IGL in a certain visual field (counting grid). The same approach was used for the TUNEL-positive nuclei. The statistical significance of the differences was calculated with the Mann-Whitney ($P < 0.01$) and Kruskal-Wallis (χ -square above 20) tests and error bars in Fig. 5 indicate one s.d.

Detection of β -galactosidase activity

Adult brains were dissected out into cold PBS, cooled at 4°C for 5

Table 1. The number of analyzed mice of different compound genotypes at time points relevant for cerebellar development

Genotype	Age				
	P1	P8	P15	P20	P35
Control	4	4	3	3	1
<i>p107^{-/-}</i>	4	3	6	5	2
<i>En2 cre; Rb^{LoxP/LoxP}</i>	1	3	3	3	2
<i>En2 cre; Rb^{LoxP/LoxP}; p53^{+/-}</i>	–	–	1	–	–
<i>En2 cre; Rb^{LoxP/LoxP}; p53^{-/-}</i>	–	–	1	–	–
<i>En2 cre; Rb^{LoxP/LoxP}; p107^{+/-}</i>	5	6	9	3	4
<i>En2 cre; Rb^{LoxP/+}; p107^{+/-}</i>	3	–	3	3	3
<i>En2 cre; Rb^{LoxP/LoxP}; p107^{-/-}</i>	3	3	3	4	4
<i>En2 cre; Rb^{LoxP/+}; p107^{-/-}</i>	3	–	4	–	2
<i>En2 cre; Rb^{LoxP/LoxP}; p107^{+/-}; p53^{-/-}</i>	–	1	3	–	–
<i>En2 cre; Rb^{LoxP/LoxP}; p107^{-/-}; p53^{+/-}</i>	–	1	–	–	–
<i>En2 cre; Rb^{LoxP/+}; p107^{+/-}; p53^{+/-}</i>	–	–	–	–	3

Thick lines indicate cerebella with histological abnormalities (no data available for time points with broken lines). In addition to the littermate controls indicated in the table, numerous single mutant mice, such as *En2-cre*, *Rb^{LoxP/+}* or *Rb^{LoxP/LoxP}* were analyzed and showed no cerebellar abnormalities.

minutes and sliced coronally or sagittally. The slices were fixed in 4% PFA/PBS at 4°C for 2 hours. The β -galactosidase staining was carried out according to standard procedure.

Sections were paraffin wax embedded and processed as described below.

Histological analysis

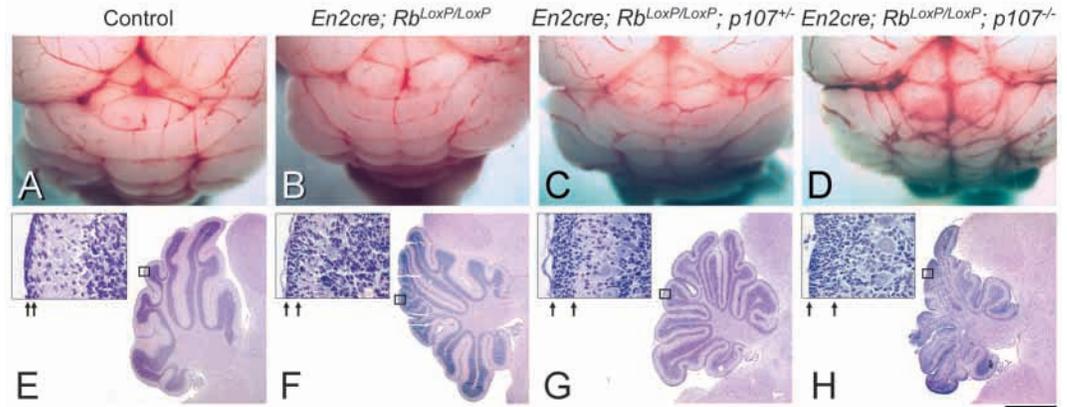
Cytospins were thawed, dried at room temperature and fixed for 10 minutes in 4% paraformaldehyde in PBS. Whole mouse brains were fixed for at least 12 hours in 4% buffered paraformaldehyde. Coronal or sagittal slices were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 4 μ m nominal thickness were mounted on coated slides and routinely stained with Haematoxylin and Eosin (H&E). Immunohistochemistry for Gfap (polyclonal, 1:300, DAKO), calbindin-D 28K (monoclonal, 1:400 Sigma), parvalbumin (polyclonal, 1:300 Swant, CH), NeuN (Neuna60 – Mouse Genome Informatics) (monoclonal, 1:4000 Chemicon), p27 (polyclonal, 1:400 SantaCruz), BrdU (monoclonal, 1:50 DAKO), TuJ-1 (Tubb3 – Mouse Genome Informatics) (monoclonal 1:20, Abcam) and anti phosphorylated histone H3 (polyclonal, 1:200 Upstate Biotechnology) was performed on selected sections. A microwave pre-treatment with 0.01M citrate buffer (pH 6.0) was used for p27, NeuN, anti-phosphorylated histone H3 and BrdU; Proteinase K digestion (20 μ g/ml, Roche for 20 minutes at 20°C) was used for calbindin-D 28K. Biotinylated secondary antibodies (goat anti-rabbit and rabbit anti-mouse, DAKO) were used at a dilution of 1:200. Visualization was achieved using biotin/avidin-peroxidase (DAKO) and diaminobenzidine as a chromogen.

RESULTS

Region-specific inactivation of Rb during cerebellar development: structural defects, delayed terminal differentiation and loss of specific cell types

To achieve a region selective inactivation of Rb in the cerebellum that encompassed all cell types during development and in adulthood, we used engrailed2-Cre (*En2cre*) transgenic mice (Zinyk et al., 1998). These mice have been shown to express Cre at embryonic day (E) 9.5 specifically in the dorsal mid-hindbrain junction. These mice were crossed to mice

Fig. 1. Cerebellar phenotype of Rb and Rb/p107 compound mutant mice at P15. Gross appearance of the cerebellum of wild type (A) and mutant (B-D) mice at postnatal day 15. Note the reduced size of the double mutant vermis. (E-H) Hematoxylin and Eosin stained median sagittal sections through the cerebellum of *En2cre*; *Rb^{LoxP/LoxP}* mice ($n=3$) (F) show preserved foliation, but slight reduction of the vermis size and retarded migration of granule cells (inset, arrows indicate EGL thickness), when compared with a wild-type littermate ($n=3$) (E). A similar, but more pronounced phenotype was observed in the cerebella of Rb/p107 double mutant mice (*p107^{+/-}*; $n=9$, *p107^{-/-}*; $n=3$) (G,H). Scale bar: 1 mm (100 μ m in insets).



carrying conditional Rb alleles (Marino et al., 2000; Vooijs and Berns, 1999). Their recombination results in truncation of the Rb protein that is functionally equivalent to a null allele.

Both *En2cre*; *Rb^{LoxP/+}* and *En2cre*; *Rb^{LoxP/LoxP}* mice were healthy and fertile and showed no indications of cerebellar malfunction. We examined the cerebella of these mice at time points pivotal for cerebellar development (P1, P8, P15) and at days P20, P35 and P180 (6 months, Table 1).

No macroscopic or histological differences between wild-type and *En2cre*; *Rb^{LoxP/LoxP}* mice were observed at postnatal days 1 and 8. Although at P15 the outer appearance of mutant brains was not much different from wild type cerebella (Fig. 1A,B), the thickness of the EGL remained greater in *En2cre*; *Rb^{LoxP/LoxP}* mice (four or five cells thick, Fig. 1F) when compared with those of wild-type littermates (one or two cells thick, Fig. 1E).

At P20, when the cerebellar cortex has attained its mature structure, it consists of (1) an outer molecular layer with the dendritic arbors of the Purkinje cells, parallel fibers of granule cells, stellate and basket interneurons and processes of the Bergmann glial cells; (2) the Purkinje cell layer and nuclei of Bergmann glia; and (3) an inner layer containing mainly granule cells and few Golgi neurons (Fig. 2A-E). The most striking findings in *En2cre*; *Rb^{LoxP/LoxP}* mice were a persistent thin EGL with NeuN negative but TuJ1 (Class III β tubulin) positive granule cell precursors (Fig. 2F,G) still migrating through the molecular layer and clearly reduced thickness and cellularity of the IGL, which was most pronounced in lobules VI and VII (Fig. 2A,F), and far less apparent in lobules I-V and VIII-X. Purkinje cells were instead well differentiated, as estimated by immunostaining for calbindin D 28K (Fig. 2H) and parvalbumin (Fig. 2I), but slightly irregularly arranged and showed moderately enlarged nuclei and somata (Fig. 2H,I). However, parvalbumin staining revealed that the interneurons of the molecular layer, which are the only additional parvalbumin-expressing cells of the cerebellum, were substantially reduced in number (Fig. 2I). The neuronal loss in the IGL was confirmed in the NeuN immunostaining (Fig. 2F) and was accompanied by mild astrogliosis within the IGL (Fig. 2J). At P35, a progressive loss of granule cells led to a mild shrinkage of the vermis, which was particularly pronounced in lobules VI and VII. Purkinje cells, albeit largely preserved

appeared disarranged, occasionally showed enlarged nuclei with abnormal shapes and reduced dendritic arborization. The latter finding probably resulted from the persistent EGL. A brisk widespread astrogliosis was observed in the IGL.

These results suggest that granule precursor cells lacking Rb achieve a proper, though delayed, terminal differentiation, and correctly migrate in vivo during development. However, a fraction of the granule cell population exhibited a short life span and degenerated rapidly. Interestingly, not all cerebellar cell populations were equally affected by Rb depletion. Although the number of granule, basket and stellate neurons was severely reduced, Purkinje cells, though morphologically abnormal, were still present in the adult cerebellum and astrocytes did not appear to be particularly affected by the lack of Rb.

Lack of Rb in all cerebellar cell types located in the vermis of *En2cre*; *Rb^{LoxP/LoxP}* mice

It could be argued that the survival of Purkinje cells was due to incomplete Cre-mediated recombination and retained Rb expression. We therefore crossed *En2cre* transgenic mice with the *ROSA26* reporter mice (Ziyk et al., 1998) in which Cre-mediated recombination results in removal of a Stop cassette and transcription of a β -galactosidase gene. *lacZ* expression was found in a region largely corresponding to the vermis (Fig. 3A-C) and immunostaining with calbindin and NeuN confirmed that *lacZ* colocalized in granule neurons and Purkinje cells, respectively (Fig. 3D,E). To exclude that a subpopulation of Purkinje or granule neurons, undetected by β -galactosidase staining, remained unrecombined, we additionally performed cell separation and PCR recombination analysis on cell fractions.

In a two-step Percoll gradient, cerebellar cells were separated according to their size in two fractions and then identified morphologically and immunohistochemically on cytospin preparations (Fig. 3F-H). Fraction 1 contained astrocytes and Purkinje cells (Fig. 3F,G), while Fraction 2 mainly granule cells and granule cell progenitors (Fig. 3H). PCR recombination analysis of both fractions confirmed the absence of unrecombined cells in the vermis of *En2cre*; *Rb^{LoxP/LoxP}* mice (Fig. 3I, lanes 5, 6). We therefore conclude that all cells located in the cerebellar vermis are effectively depleted of Rb in *En2cre*; *Rb^{LoxP/LoxP}* mice.

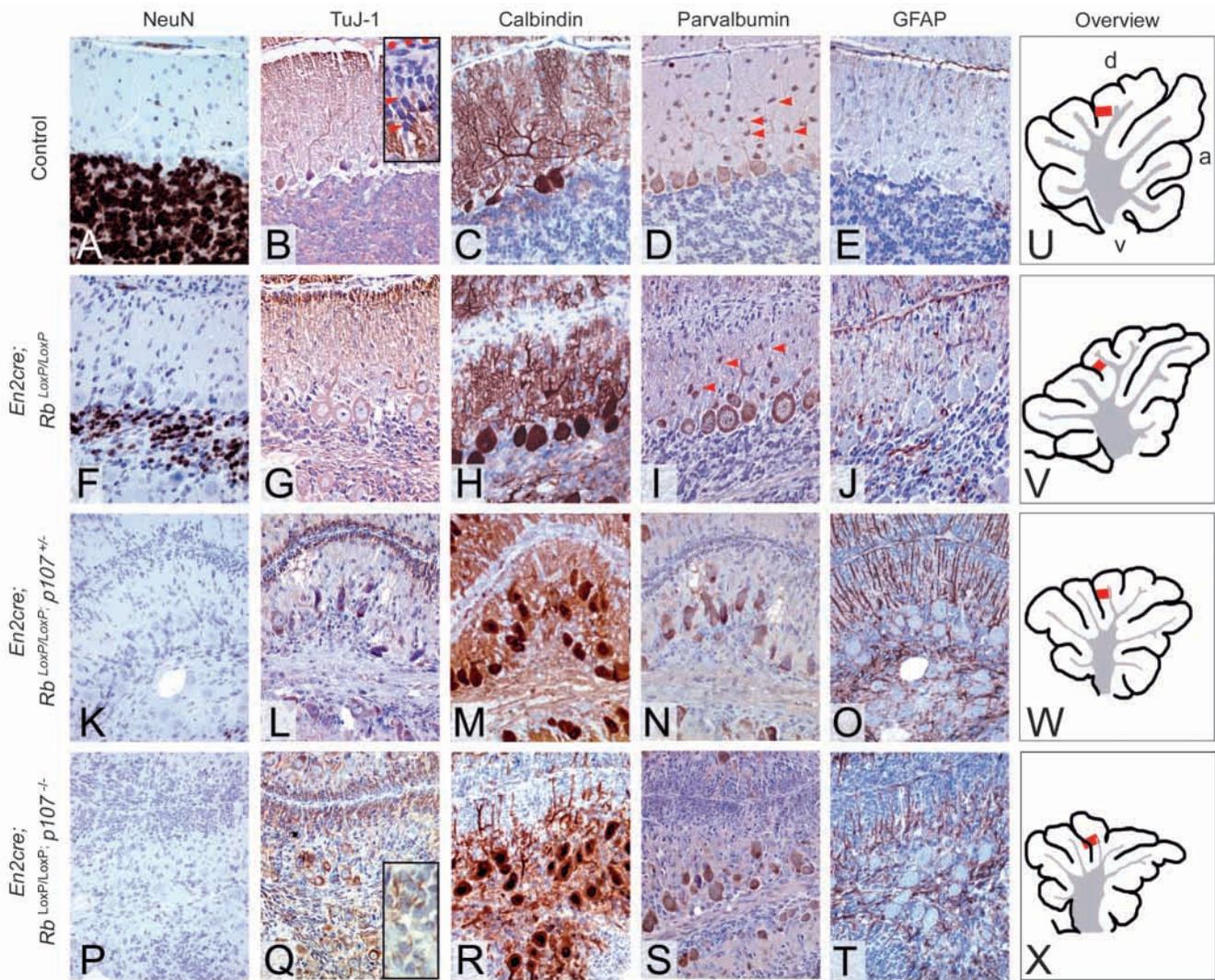


Fig. 2. Analysis of the cerebellar phenotype of Rb and Rb/p107 compound mutant mice. Sagittal sections of wild-type (A-E) ($n=3$), Rb (F-J) ($n=3$) and Rb/p107 compound mutant (K-T) ($p107^{+/-}$; $n=3$, $p107^{-/-}$; $n=4$) cerebella at P20. NeuN immunostaining (A,F,K,P) reveals marked loss of granule cells in the Rb-deficient vermis (F), while additional loss of one or both p107 alleles results in impaired terminal differentiation and in almost complete granule cells loss (K,P). Aberrantly migrating neurons can still be detected by immunostaining for TuJ1 (B,G,L,Q). Inset in B shows expression of TuJ1 in the inner postmitotic, pre-migratory cell population (arrowheads), but not in the outer layers of the EGL in a P8 control mouse. Circles indicate the outer margin of the cerebellar folium. In L,Q, there is still a TuJ1-expressing cell population in the EGL present. Inset in Q shows a higher magnification of TuJ1-expressing cells above the level of dispersed Purkinje cells. Calbindin-positive Purkinje cells (C,H,M,R) are increasingly disarranged with dystrophic dendrites in Rb-deficient (H) and in Rb/p107 double mutants (M,R). Likewise, parvalbumin antiserum stains Purkinje cells (D,I,N,S) but also reveals a loss of interneurons of the molecular layer (arrowheads in D,I). Dystrophic Bergmann glia and an increasing astrogliosis are highlighted by GFAP immunostaining (E,J,O,T). (U-X) Representative drawings of midsagittally cut cerebella with indication of the areas (red square) shown at high magnification in A-T. Scale bar: 100 μ m in A-T.

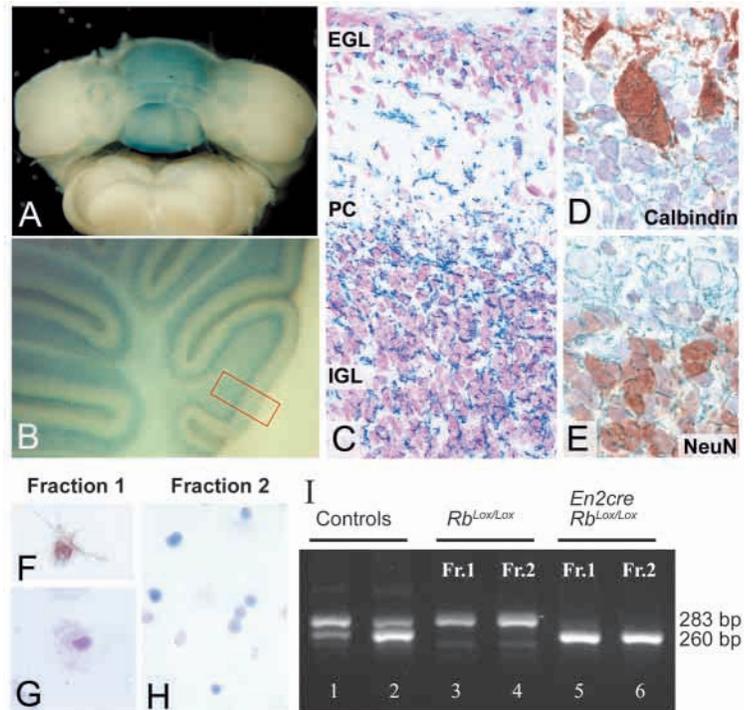
Impaired terminal differentiation and migration of granule cell precursors lacking Rb and p107

The pocket protein family member p107 is expressed from E10.5 in the ventricular zone of the neuroepithelium, which contains proliferating neuronal precursor cells (Jiang et al., 1997). p107 is rapidly downregulated when cells undergo terminal differentiation. By contrast, the third pocket protein family member, p130, is expressed at low levels in precursor and mature neurons. In vitro studies have shown that Rb-deficient neural precursor cells exhibit little change in p130 levels but show a marked increase in the amount of p107

protein during neurogenesis (Callaghan et al., 1999; Lipinski and Jacks, 1999), implying that p107 might substitute for Rb in facilitating differentiation.

To assess a possible overlap between Rb and p107 function in the developing cerebellum, we examined the effects of *En2cre* mediated *Rb* inactivation in a *p107*-null background. We crossed *En2cre*; *Rb*^{LoxP/LoxP} mice with *p107*-deficient mice (Robanus-Maandag et al., 1998) and obtained *En2cre*; *Rb*^{LoxP/LoxP}; *p107*^{+/-}, *En2cre*; *Rb*^{LoxP/+}; *p107*^{-/-} and *En2cre*; *Rb*^{LoxP/LoxP}; *p107*^{-/-} compound mutant mice (Table 1). Mice hemizygous or nullizygous for p107 do not show CNS

Fig. 3. Recombination analysis in the vermis of *En2cre; Rb^{LoxP/LoxP}* mice. (A,B) Whole-mount β -galactosidase staining of the vermis of *En2cre; ROSA26^{LoxP}* indicator mice shows the area of recombination from outside (A) and after sagittal sectioning (B). (C) Histology of an area corresponding to the red square in B (Nuclear Red counterstaining; EGL, external granular layer; PC, Purkinje cells; IGL, internal granular layer), demonstrating the *lacZ* expression in all areas of the cerebellum. Adjacent sections were immunostained for calbindin (D) or NeuN (E) to confirm the *En2cre*-mediated recombination in these cell populations. (F-I) PCR analysis of cell fractions obtained from Percoll gradients. Fraction 1 contains larger cells (astrocytes, GFAP immunostaining in F; Purkinje cells, Hematoxylin and Eosin staining in G), while Fraction 2 contains small cells, such as granule cells and granule cell precursors (Hematoxylin and Eosin staining in H). (I) PCR recombination analysis of both fractions shows a 283 bp product representing the floxed allele (primers Rb19E and Rb18) and a 260 bp product of recombined Rb allele (primers Rb212 and Rb18). Genomic DNA extracted from Percoll-separated cerebellar fractions of *Rb^{LoxP/LoxP}* and *En2cre; Rb^{LoxP/LoxP}* mice were used in lanes 3,4 and 5,6, respectively. Lanes 3 and 5, larger size fraction 1. Lanes 4 and 6, lower size fraction 2. Two controls with partial recombination are shown in lanes 1 and 2.



abnormalities (Lee et al., 1996; Robanus-Maandag et al., 1998). In addition, loss of one Rb allele in the vermis of p107 hemizygous or nullizygous mice (*En2cre; Rb^{LoxP/+}; p107^{-/-}*) did not result in any abnormality even at 26 weeks of age.

We then obtained mice hemizygous or nullizygous for *p107* in which both Rb alleles were recombined in the vermis (*En2cre; Rb^{LoxP/LoxP}; p107^{+/-}*, *En2cre; Rb^{LoxP/LoxP}; p107^{-/-}*, Table 1). Both compound mutants developed ataxia between P15 and P20. Macroscopic examination at P15 revealed a severe reduction of the vermis size, which was more evident if both *p107* alleles were lost (Fig. 1C,D). In keeping with the severity of neurological signs and prominence of macroscopic findings, all histopathological abnormalities were more pronounced in *En2cre; Rb^{LoxP/LoxP}* mice that were nullizygous for *p107* than in those hemizygous for *p107* (Fig. 1G,H).

At P20, *Rb* and *p107* double mutant mice showed highly disorganized cerebellar architecture of the median cerebellar region (Fig. 2). The EGL was still six or seven cells thick and the molecular layer appeared clearly reduced in size (Fig. 2K,P). EGL cells albeit negative for NeuN were clearly positive for the early neuronal marker TuJ1 (Fig. 2L,Q). Purkinje cells were terminally differentiated, as estimated by the observation of their dendritic tree reaching the pial surface in the immunostaining for calbindin (Fig. 2M,R). However, the dendritic arborization appeared shrunk, with stunted to misoriented dendrites (Fig. 2M,R) and the PC bodies appeared to be poorly aligned with substantial loss of laminar distribution. These abnormalities were at least partially due to the presence of the thick EGL and the impaired formation of an IGL. Occasionally, binucleated Purkinje neurons were observed. Focally, clusters of small, NeuN-negative but TuJ1-positive granule cells were detected in the rudimentary molecular layer and intermingled with Purkinje cells. GFAP immunostaining revealed dystrophic

Bergmann glia cells and a marked astrogliosis in the IGL (Fig. 2O,T).

Two weeks later in postnatal development, at day 35, we found a complete loss of granule cells in the IGL, which now mainly contained reactive astrocytes. Surprisingly, Purkinje cells were still preserved, though there was a substantial degree of disarrangement, ballooning of the nucleus and fragmentation of chromatin.

We conclude that p107 can partially compensate for the lack of Rb in promoting differentiation and migration. However, p107 only partially restores normal cell cycle exit. In the absence of Rb and p107, no other pocket protein family member, i.e. p130 or a yet unknown member, takes over the function in granule cells. Instead, Purkinje cell and astrocyte development and differentiation does not appear to be crucially dependent on Rb and p107 function.

Rb and p107 are not required for Purkinje cell differentiation and survival

Purkinje cells in *En2cre; Rb^{LoxP/Lox}* and *En2cre; Rb^{LoxP/Lox} p107* compound mutant mice showed enlarged soma size, thickened processes and bizarre nuclear shape. In addition, they were irregularly arranged, in particular upon loss of p107. Loss of appropriate environment can cause abnormalities in neuronal morphology and may even lead to cell death. To dissect whether the above effects were intrinsic to Purkinje cells or rather a consequence of improper environment, and whether the phenotype could be rescued in an environment expressing Rb and p107, we ablated Rb either alone or in combination with p107 selectively in Purkinje cells. To achieve this, we crossed L7cre transgenic mice (Marino et al., 2002) with *Rb^{LoxP}* and with *p107^{-/-}* mice to assess the role of Rb and p107 in Purkinje cell development and survival. Neither deletion of Rb alone or in combination with p107 resulted in

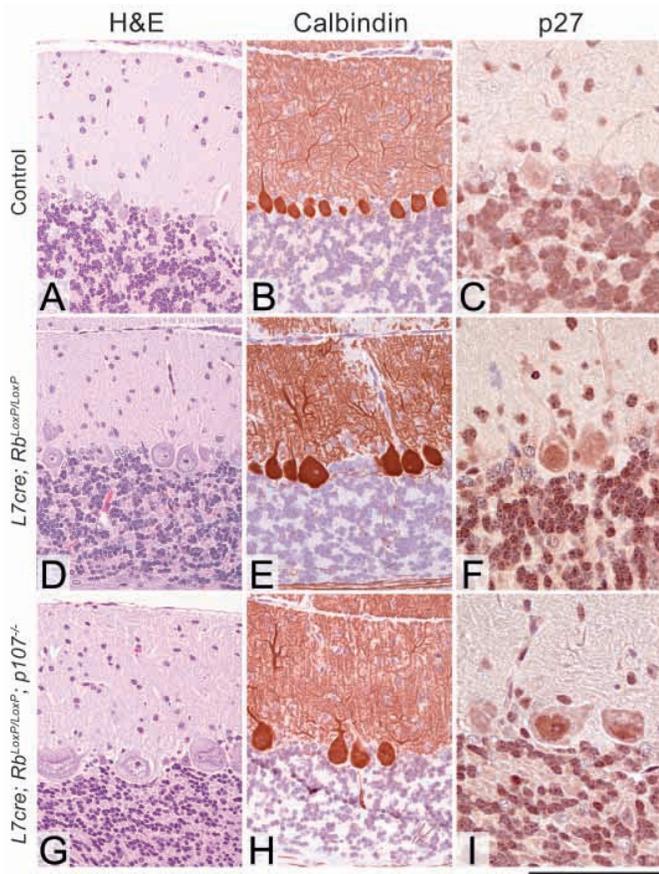


Fig. 4. Purkinje cell-specific inactivation of Rb and p107 at P30. Purkinje cells are enlarged, show bizarre nuclei, and are occasionally displaced into the molecular layer (D,G) when compared with control (A). Calbindin staining shows a largely intact dendritic arborization in the molecular layer (E,H), similar to wild-type cerebella (B). The cell cycle inhibitor p27 is accumulated in the nucleus of most Rb- or Rb/p107-deficient Purkinje cells (F,I), when compared with wild-type cells (C). Controls, $n=2$; $L7cre;Rb^{LoxP/LoxP}$, $n=3$; $L7cre;Rb^{LoxP/LoxP};p107^{-/-}$, $n=3$. Scale bar: 100 μm in A,B,D,E,G,H; 200 μm in C,F,I.

neurological deficits until 6 weeks of age. Histological examination of three $L7cre; Rb^{LoxP/Lox}$ and three $L7cre; Rb^{LoxP/Lox} p107^{-/-}$ at postnatal day 20 revealed regular cerebellar architecture and no apparent loss of Purkinje cells when compared with age- and genetic background-matched controls. However, as we did not count Purkinje cells, we cannot exclude a minor cell loss. Purkinje cells were greatly enlarged, occasionally slightly displaced towards the molecular layer and exhibited enlarged nuclei with abnormal shapes, resembling hourglass or kidney (Fig. 4D,E,G,H). These experiments suggest that the Purkinje cell phenotype is largely cell-autonomous and cannot be rescued by adjacent, Rb- and p107-expressing cells. Additionally no delayed differentiation and migration and no abnormal proliferation and apoptosis of granule cells were observed. Therefore, Purkinje cell dysplasia in $En2cre; Rb^{LoxP/Lox}; p107^{-/-}$ mice is a result of Rb and p107 loss of function, rather than an effect of improper positioning or loss of surrounding granule cells. Moreover the granule cell defects observed in $En2cre; Rb^{LoxP/Lox}; p107^{-/-}$ mice are not secondary to Purkinje cell abnormalities.

Rb and p107 in proliferation and apoptosis in the cerebellum

First, we assessed the proliferation rate of granule cell precursors lacking Rb or Rb and p107 at postnatal days 1, 8, 15 and 20 by BrdU incorporation. BrdU-positive nuclei are normally present in the granule cell precursors of the EGL up to postnatal day 15 (Fig. 5H) and occasionally in scattered astrocytes of the IGL. Proliferating cells were counted in compound mutants and controls and labeling indexes were calculated. Although there was no difference at P1 and P8, a considerably higher proliferation in Rb mutant cerebella was found at P15 (Fig. 5A,C) and at P20 in the EGL. In the Rb mutant IGL, however, a substantial increase of proliferating granule cells was detected as early as postnatal day 8. In $Rb/p107$ double mutant cerebella, these findings were even more pronounced than in Rb-deficient vermis (Fig. 5A,C,E,H). All BrdU staining results were confirmed by immunostaining for phosphorylated histone H3 (data not shown), which has been shown to correlate to chromatin condensation at mitosis (Juan et al., 1998).

We then examined the status of the CDK inhibitor $p27^{Kip1}$, which is part of a cell-intrinsic timer that arrests the cell cycle and initiates differentiation in various cell types (Durand et al., 1998), among them neural cells. p27 expression is inversely correlated to the proliferative status of cells. At P15, wild-type IGL neurons and the inner portion of the EGL showed strong nuclear p27 expression, while granule cell precursors in the outer EGL of the molecular layer were p27 negative (Fig. 5F). Instead, Rb depleted EGL cells of both regions expressed almost no p27 (Fig. 5I) and most of the granule cells that have left the EGL and have migrated towards the IGL were negative, too. As p27 is expressed only in differentiating postmitotic neural precursors, the findings here are in keeping with the observation that Rb-deficient EGL precursors and IGL neurons are still actively and abnormally cycling.

Although granule cells are mitotically active during postnatal cerebellar development, Purkinje cells are among the first neurons to leave the ventricular zone, become postmitotic and migrate to form a temporary plate-structure during cerebellar development between E11 and E13. It has been demonstrated that inactivation of Rb family members in Purkinje cells through a cell-specific expression of a modified T-antigen (Feddersen et al., 1995), which was modified not to interact with p53, leads to ectopic Purkinje cell proliferation and apoptosis. We therefore assessed whether Purkinje cells lacking Rb or Rb and p107 showed abnormal proliferation and apoptosis.

Both, $En2cre$ - and $L7cre$ -mediated inactivation of Rb alone or in a $p107$ -null background, resulted in terminal differentiation of Purkinje cells, as estimated by calbindin and parvalbumin expression (Fig. 2H,M,R; Fig. 4E,H), and in normal migration from the ventricular zone of the neuroepithelium and formation of a well-defined monolayer. From P15 onwards, a considerable number of Purkinje cells showed enlarged soma size, bizarre nuclei and occasional fragmentation of chromatin. After BrdU incorporation, single Purkinje cells lacking Rb or Rb/p107 displayed nuclear BrdU staining, whereas BrdU labeling was never observed in Purkinje cells of control littermates. These results were confirmed by staining for phosphorylated histone H3 (data not shown). However, while bizarre nuclei and even occasional

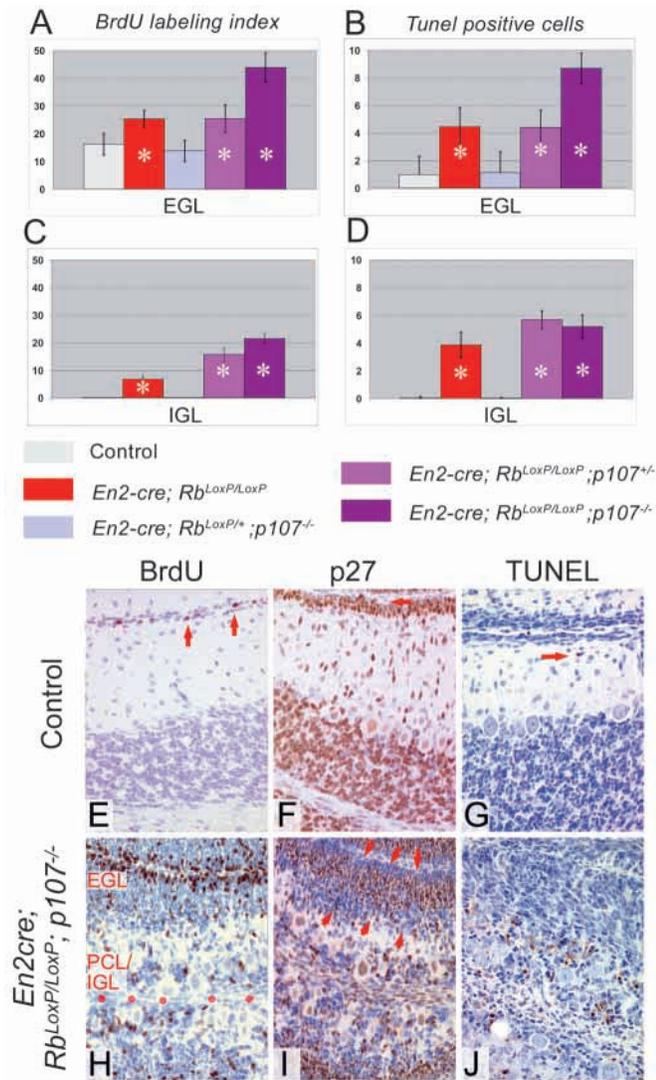


Fig. 5. Proliferation and apoptosis in cerebella lacking Rb or Rb/p107 at P15. Proliferation (assessed by incorporation of BrdU) in the EGL (A) and IGL (C) is increased in the Rb- and p107-deficient vermis, but is counterweighted by an increased apoptosis rate in the vermis deficient of Rb and Rb/p107 (B,D). Proliferation and apoptosis were most pronounced if both Rb and p107 alleles were lost. Asterisks indicate that the value is significantly different from control value ($P < 0.01$ for all values). (D) There is no statistically significant difference between *En2cre; Rb^{LoxP/LoxP}; p107^{+/-}* (purple bar) and *En2cre; Rb^{LoxP/LoxP}* (red bar). Standard deviations (± 1 s.d.) are indicated by error bars. (E,H) BrdU labeling of P15 wild-type (E; arrows indicate proliferating cells in the EGL) and Rb/p107 double mutant (H) illustrated the markedly increased proliferation in the broadened EGL and in the IGL. The cell layers in the double mutant (H) are indicated (EGL, external granular layer; PCL, Purkinje cell layer; IGL internal granular layer). Red circles indicate the inner border of the presumed IGL, which would normally form the cerebellar white matter. The cell cycle inhibitor p27 is expressed in cells of the inner EGL, in most migrating and IGL neurons (F). The outer p27-negative EGL region is broadened in Rb/p107 double mutant cells (red arrow in F indicates the layer that is one or two cells thick; arrows in I indicate the broadening of the p27-negative outer EGL). Likewise, a substantial proportion of migrating and IGL neurons are p27 negative (arrows, I). As indicated by the graphs (B,D), there is also significantly increased apoptosis in double mutant (J) compared with wild-type (G) vermis. Arrow in G indicates a single TUNEL-positive cell. Scale bar: 100 μ m.

binucleated Purkinje cells were observed, there were no unambiguous cell divisions found. Interestingly, a large fraction of Purkinje cells deficient for Rb or Rb/p107 showed a strong nuclear p27 accumulation, suggesting a mechanism counteracting cell division (Fig. 4F,I).

Cerebella of wild-type and *Rb/p107* double mutant mice were indistinguishable in size, morphology and proliferation rate at P1, but reduction in size and architectural abnormalities became obvious at P15 (Fig. 1), indicating that the cell reduction takes place before complete development of the cerebellum. To assess granule cell death, we performed the TUNEL assay on cerebellar sections at P1, P8, P15 and P20. At P1 and P8, few apoptotic cells were seen in the still actively proliferating EGL and in the postmitotic IGL of wild-type mice. No difference in apoptotic rate was detected in double mutant cerebella mice at P1 but increased by P8. To quantify and compare the results, positive nuclei in EGL and IGL layers were separately counted and labeling indexes were calculated. A trend towards a slightly higher apoptotic index was observed at P8 but did not reach statistical significance. At P15, however, a dramatic increase of the apoptotic rate occurred in the vermis deficient for Rb or Rb and p107 (Fig. 5B,D,G,J; Fig. 6B). Most

positive cells were found in the inner half of the EGL and in the IGL (Fig. 5J; Fig. 6B). Similar apoptotic rates were found up to P30 when the granule cell loss was almost complete. Although differentiation still occurred in granule cells lacking Rb, the majority of these cells failed to exit cell cycle and underwent apoptosis within in the inner region of the EGL or after migration towards the IGL. The additional lack of p107 accelerated and aggravated the process.

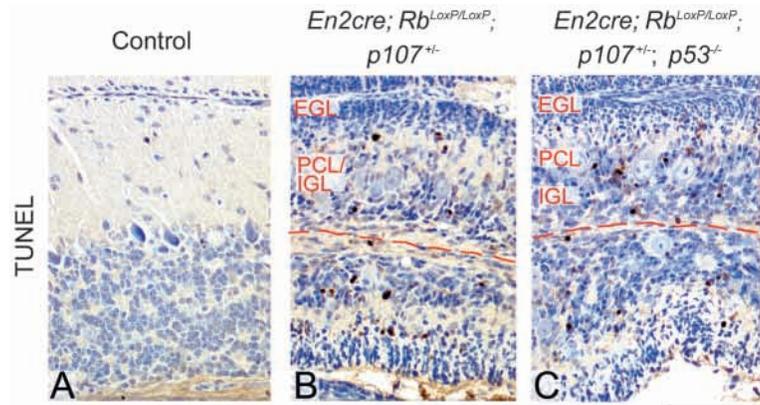
In contrast to the massive apoptosis of granule cells, there was no noticeable Purkinje cell apoptosis in *L7cre* or *En2cre* Rb/p107 compound mutants. To assess the effect of long-term inactivation of pocket proteins in Purkinje cells, we kept *En2cre; Rb^{LoxP/LoxP}; p107^{-/-}* and *L7cre; Rb^{LoxP/LoxP}* mice for 4 and 6 months, respectively. Inactivation of Rb and p107 in the vermis resulted in a complete loss of granule cells, a brisk astrogliosis but only mild reduction of Purkinje cells. Likewise, long-term ablation of Rb selectively in Purkinje cells resulted in mild cell loss and subtle rarefaction of the dendritic arborization.

These results indicate that Purkinje neurons achieve terminal differentiation in the absence of Rb or Rb and p107. Like granule cells, Purkinje neurons incorporated BrdU and expressed markers that suggest cell cycle activity well beyond the time they should have become postmitotic, though this did not elicit programmed cell death, as was the case in granule cells.

Apoptosis of ectopically proliferating granule cells lacking Rb is not p53 mediated

Cell death in Rb mutant embryos has been shown to be p53 dependent in the developing central nervous system (Macleod et al., 1996) and in the lens (Liu and Zacksenhaus, 2000; Morgenbesser et al., 1994). To determine whether apoptosis of abnormally proliferating, terminally differentiated granule

Fig. 6. Lack of p53 does not rescue cerebellar neurons deficient for Rb and p107. TUNEL staining of control (A), *En2cre; Rb^{LoxP/LoxP}; p107^{+/-}* (B) and *En2cre; Rb^{LoxP/LoxP}; p107^{+/-}; p53^{-/-}* (C) cerebella at postnatal day 15 shows no difference in the extent of apoptosis upon loss of p53. The cell layers in the double mutant (B,C) are indicated (EGL, external granular layer; PCL, Purkinje cell layer; IGL internal granular layer). Broken red lines indicate the inner border of the presumed IGL, which would normally form the cerebellar white matter. The cerebellum of a wild-type littermate is shown on the left as a control (A). Scale bar: 50 μ m.



neurons lacking Rb is dependent on p53, we generated animals lacking Rb in cerebellar precursor cells and hemizygous for p107 in a *p53^{-/-}* background (*En2cre; Rb^{LoxP/LoxP}; p107^{+/-}; p53^{-/-}*) and examined the cerebella at postnatal day 15, a time point characterized by a brisk apoptosis in *En2cre; Rb^{LoxP/LoxP}; p107^{+/-}*. The percentage of TUNEL-stained nuclei as well as the percentage of BrdU-labeled cells in the EGL and IGL of compound mutant mice was similar to that in *En2cre; Rb^{LoxP/LoxP}; p107^{+/-}* mice (Fig. 6). The structural and morphological abnormalities observed in double mutants were comparable with single mutant mice. Similar results were obtained when comparing proliferation and apoptotic labeling indexes in postnatal day 15 cerebella of *En2cre; Rb^{LoxP/LoxP}* and *En2cre; Rb^{LoxP/LoxP}; p53^{-/-}* mice (Fig. 6 and data not shown). We conclude that loss of p53 function does not suffice to rescue Rb deficient neural precursors from apoptosis. This is important in light of previous evidence showing that apoptosis is p53-dependent in the CNS of developing *Rb^{-/-}* embryos (Macleod et al., 1996). Our results demonstrate the critical contribution of p53-independent pathways in apoptosis occurring in the developing central nervous system after Rb loss.

DISCUSSION

The best-characterized role of the pocket proteins is the regulation of the cell cycle, which is mainly exerted through their interaction with the E2f family of transcription factors (reviewed by Harbour and Dean, 2000). A function in protecting cells from apoptosis and in induction of differentiation during development has emerged from the phenotypic analysis of *Rb^{-/-}* embryos (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The impaired development of the central nervous system of *Rb^{-/-}* embryos is characterized by lack of cell cycle exit, elevated apoptosis and failure to attain a fully differentiated state (Lee et al., 1994). Thus far, the analysis has been limited to E13–15 embryos, which also showed severe defects in fetal liver function, suggesting that the CNS phenotype might have been precipitated by the defect in hematopoiesis. Studies in chimeric mice have shown a normal contribution of *Rb^{-/-}* ES cells to the CNS, suggesting rescue through paracrine mechanisms (Maandag et al., 1994; Williams et al., 1994) although cerebellar granule neurons and Purkinje cells were showing morphological abnormalities and

cell loss. Recently, conditional inactivation of Rb in forebrain precursor cells showed that Rb deficient neurons are able to survive, leading to enhanced neurogenesis and increased telencephalic size (Ferguson et al., 2002). However, early postnatal lethality of this conditional mutant has limited the further analysis of the fate of these cells. In vitro studies suggest that cortical neural precursor cells lacking Rb show delayed terminal mitosis but full differentiation and survival in vitro (Callaghan et al., 1999).

To study the role of Rb and p107 in cerebellar development, we inactivated *Rb* or *Rb/p107* either in precursor cells of the vermis region or selectively in Purkinje cells. As our mouse models were not limited by postnatal death, we were able to dissect the role of Rb and p107 in development, differentiation and survival of all cerebellar cell populations.

We show that cerebella lacking one *Rb* allele and both *p107* alleles were indistinguishable from control littermates: cell cycle proteins were upregulated in proliferating cells located in the outer zone of the EGL, whereas cells in the inner zone of the EGL have stopped DNA synthesis and started differentiation and migration towards the IGL as it is the case in wild-type mice. This was paralleled by upregulation of cell cycle inhibitors, such as p27 (Miyazawa et al., 2000). By contrast, the lack of Rb resulted in several cell cycle abnormalities in granule cell precursors, which were even more pronounced if one or both *p107* alleles were lost. The cell cycle marker p-histone H3 was expressed throughout the entire EGL, which was reflected by an increased BrdU incorporation rate, while the expression of the cell cycle inhibitor p27 was reduced in these cells, suggesting that Rb and *Rb/p107* deficient EGL cells cannot properly exit the cell cycle. The presence of IGL neurons (as indicated by the continued expression of TuJ1) expressing proliferation markers and incorporating BrdU can be explained by the inability of Rb-deficient granule cell precursors to switch to a postmitotic state. However, BrdU incorporation and expression of cell cycle markers in these granule cells might as well represent an unscheduled re-entry into cell cycle before death as described by (Herrup and Busser, 1995). Our results clearly show that loss of Rb function is detrimental for controlled differentiation, cell cycle exit and survival of cerebellar granule cell precursors, and that a single functional *Rb* allele is sufficient to impose a completely normal phenotype to the cerebellum of the mutant mice even in the complete absence of p107.

An essential involvement of Rb in the terminal

differentiation process of these precursors, as has been described for myoblasts (Novitch et al., 1999) and adipocytes (Classon et al., 2000), seems unlikely because differentiating granule cells express mature neuronal markers (our data) (Ferguson et al., 2002). The lack of expression of mature neuronal markers described by Lee et al. in *Rb*^{-/-} embryos might have been caused by secondary neural precursor damage as a result of the severe defects in hematopoiesis (Lee et al., 1992). Callaghan and co-workers have suggested that p107 might substitute for Rb function to facilitate differentiation in vitro, as they detected enhanced levels of p107 but not of p130 during neurogenesis in studies with Rb-deficient neural precursor cells (Callaghan et al., 1999). In agreement with these findings, we report here that p107 does show activities that overlap with those of Rb in promoting cell differentiation and migration of granule cell precursors in compound mutant mice. However, at physiological levels, p107 by itself is incapable of commanding exit from the cell cycle. Purkinje cells and astrocytes instead are terminally differentiating even in the absence of both Rb and p107.

Forced proliferation of terminally differentiated neurons by the absence of Rb has been shown to induce apoptosis in embryos in vivo and in cultured cells in vitro. Most likely, this is mediated by the de-repression of promoters by the loss of bound Rb/E2f complexes and increased levels of free E2f, leading to upregulation of E2f-dependent promoters. Among those are several key regulators of apoptosis, such as APAF1, caspase 3 and caspase 7 (Muller et al., 2001). We show here that the function of Rb in protecting cells from apoptosis in the CNS is cell type specific. Although granule cells are forced into programmed cell death, Purkinje cells and astrocytes do not undergo apoptosis. Purkinje cells not only achieve terminal differentiation and properly migrate in the absence of Rb, but a considerable number of them also survive complete loss of granule cells. Interestingly, transgenic mice expressing, in Purkinje cells, a modified T antigen (T₁₄₇) that inactivated all members of the pocket protein family but did not interact with p53 not only showed cell cycle re-entry but also apoptosis of these neurons (Feddersen et al., 1995). As we have specifically inactivated Rb and p107, but not p130, it might be speculated that p130 can protect Purkinje cells from apoptosis, unless the T antigen mutant exerts other functions that produce apoptosis.

How can the striking difference between granule neurons and Purkinje cells in response to lack of Rb and p107 be explained? First, Purkinje cells differentiate much earlier during development, while granule cells first migrate from the rhombic lip onto the surface of the cerebellar anlage and form the EGL, where they proliferate and migrate inwards. It is therefore possible that proliferative effects mediated by loss of Rb and p107 are counteracted by other signals during Purkinje cell development, but not during the phase in which EGL precursors should exit cell cycle.

Second, it is possible, that downregulation of p27 by Rb family members is more effective in granule cell precursors than in differentiated Purkinje cells. In contrast to wild-type Purkinje cells, which were only occasionally clearly immunopositive for p27, most Rb- or Rb/p107-deficient Purkinje cells accumulated nuclear p27. Instead, granule cell precursors in the EGL and IGL neurons are negative or weakly positive for p27, in keeping with the observation that Rb upregulates p27 in an osteosarcoma cell line (Alexander and

Hinds, 2001). It is therefore conceivable that upregulation of p27 (and possibly other functionally related cell cycle regulators) prevents Purkinje cells from uncontrolled entry into the cell cycle, while EGL precursor cells continue cycling and eventually undergo apoptosis. Absence of p27 results in delayed maturation and prolonged proliferation of EGL precursors (Miyazawa et al., 2000). In line with these findings, we observe that lack of Rb and p107 correlates with reduction of p27 expression and results in a broadened EGL with increased and prolonged EGL proliferation (Fig. 5I). However, considering the established pro-apoptotic role of p27, suppression or lack of p27 alone cannot explain the propensity of EGL neurons that Rb and p107 to undergo apoptosis.

Purkinje cells have been shown to provide trophic support for granule cells and to stimulate the proliferation of EGL cells mainly through the sonic hedgehog/patched/Gli1 signaling pathway (Wallace, 1999). Indeed, analysis of mice with cerebellar defects, among them the 'staggerer' mouse (Herrup and Mullen, 1979a; Herrup and Mullen, 1979b), the lurcher mouse (Caddy and Herrup, 1991) and the L7-ADT mouse (Smeyne et al., 1995), have shown that the degeneration of Purkinje cells during embryonic or early postnatal development leads to secondary loss of granule cells.

However, Purkinje cells differentiate and survive in mice lacking granule cells such as the weaver mutant or *Math1* (*Atoh1* – Mouse Genome Informatics) knockout mice (Ben-Arie et al., 1997). In accordance with these observations, we show here that even substantial loss of granule cells does not affect Purkinje cell viability during postnatal cerebellar development. Moreover our experiments clearly show that the dramatic granule cell loss observed in *En2cre; Rb^{LoxP/LoxP}; p107^{-/-}* is cell-autonomous.

Apoptosis occurring during lens (Morgenbesser et al., 1994) and early CNS development (Macleod et al., 1996) in *Rb*^{-/-} embryos has been shown to be p53 dependent (Pomerantz et al., 1998). However, programmed cell death occurring outside the CNS, for example in the dorsal root ganglia of the *Rb*^{-/-} embryos, was shown to be p53 independent. Likewise, in our setting apoptosis in the *Rb*- and *Rb/p107*-deficient cerebellum cannot be rescued by inactivating p53. This finding demonstrates an important contribution of p53-independent apoptosis pathways in the Rb deficient CNS as it has been shown in other mouse models for granule cell death such as the lurcher mouse (Doughty et al., 2000).

In summary, our results suggest that Rb and p107 are essential for granule cell development and differentiation, while Purkinje cell differentiation and survival does not rely on Rb/p107 function. Rb/p107-deficient granule cells undergo apoptosis by a p53-independent pathway and their loss during postnatal cerebellar development does not affect Purkinje cell survival.

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