

# Unique and overlapping functions of pRb and p107 in the control of proliferation and differentiation in epidermis

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## Summary

The retinoblastoma gene product, pRb, plays a crucial role in cell cycle regulation, differentiation and inhibition of oncogenic transformation. pRb and its closely related family members p107 and p130 perform exclusive and overlapping functions during mouse development. The embryonic lethality of *Rb*-null animals restricts the phenotypic analysis of these mice to mid-gestation embryogenesis. We employed the *Cre/loxP* system to study the function of *Rb* in adult mouse stratified epithelium. *Rb<sup>F19/F19</sup>;K14cre* mice displayed hyperplasia and hyperkeratosis in the epidermis with increased proliferation and aberrant expression of differentiation markers. In vitro, pRb is essential for the maintenance of the postmitotic state of terminally differentiated keratinocytes, preventing cell cycle re-entry. However, p107

compensates for the effects of *Rb* loss as the phenotypic abnormalities of *Rb<sup>F19/F19</sup>;K14cre* keratinocytes in vivo and in vitro become more severe with the concurrent loss of *p107* alleles. p107 alone appears to be dispensable for all these phenotypic changes, as the presence of a single *Rb* allele in a *p107*-null background rescues all these alterations. Luciferase reporter experiments indicate that these phenotypic alterations might be mediated by increased E2F activity. Our findings support a model in which pRb in conjunction with p107 plays a central role in regulating epidermal homeostasis.

Key words: Mouse, Rb1, Rb11, Rb12, Epidermis, Stem cell, Differentiation, *Cre/loxP*

## Introduction

The Rb family of pocket proteins comprises three members pRb, p107 and p130 that have unique and overlapping functions in cell cycle control, differentiation and inhibition of oncogenic transformation (Classon and Harlow, 2002). Of these, *Rb* (*Rb1* – Mouse Genome Informatics) is the predominant family member found mutated in a significant number of human cancer types. Disruption of the ‘*Rb* pathway’ through direct *Rb* mutation or via mutations in upstream regulators of *Rb* function, such as in cyclin D1, *Cdk4* or *p16<sup>ink4a</sup>* is a hallmark of sporadic cancer (Sherr and McCormick, 2002; Weinberg, 1995).

*Rb* encodes a nuclear phosphoprotein that actively represses genes required for the G1-S transition mainly through the binding to members of the E2F transcription factor family (Dyson, 1998). As cell cycle progresses, pRb is phosphorylated by cyclin/CDK complexes, resulting in the release of bound E2Fs and allowing the G1 to S progression. Accordingly, forced expression of pRb leads to growth arrest, and suppresses pRb-deficient tumor growth in mice (Riley et al., 1996). pRb inactivation is further regulated by two families of CDK inhibitors (CKIs) that regulate the CDK kinase activity (Harper and Elledge, 1996).

Most of the insights into the role of the Rb family in

proliferation and differentiation have been obtained from knockout models. Mice lacking pRb die in utero displaying defects in erythroid, neuronal and lens fiber cell differentiation (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Whereas mice with null mutations in either *p107* (*Rb11* – Mouse Genome Informatics) or *p130* (*Rb12* – Mouse Genome Informatics) are viable and normal, mice lacking both *p107* and *p130* die at birth with defects in endochondral bone development associated with inappropriate cell cycle exit (Cobrinik et al., 1996; Lee et al., 1996). This indicates that p107 and p130 perform overlapping functions that cannot be carried out by pRb. Similarly, pRb may have overlapping functions with p130 that are not shared with p107 (Ciarmatori et al., 2001). Finally, mice deficient for both pRb and p107 show an aggravated *Rb*-null phenotype (Lee et al., 1996), and mouse embryo fibroblasts lacking all three Rb family members are immortal and do not respond to senescence inducing signals (Dannenberg et al., 2000; Sage et al., 2000).

The epidermis is well suited to study proliferation and differentiation as both processes are compartmentalized. The proliferative cells are confined to a single basal layer and the non-proliferative differentiating cells are located in the suprabasal layers. At their final stage of differentiation, epidermal cells are shed from the skin. This process requires a

continuous replenishment of cells, which is fulfilled by epidermal stem cells. These stem cells display a low proliferative rate and give rise to all the epidermal cell subtypes (Fuchs et al., 2001; Watt, 2001; Alonson and Fuchs, 2003).

The induction and maintenance of cell cycle arrest is crucial for normal tissue homeostasis and regulators of the cell cycle also modulate epidermal differentiation (Di Cunto et al., 1998; Paramio et al., 1998; Ruiz et al., 2003; Zhang et al., 1998; Zhang et al., 1999). Recently, the Rb family has been implicated in several aspects of differentiation processes such as terminal cell cycle exit, maintenance of the post-mitotic state and induction of tissue-specific gene expression (Lipinski and Jacks, 1999).

We and others have found a role for Rb family members during the *in vitro* differentiation of keratinocytes (Martinez et al., 1999; Paramio et al., 1998), involving the specific interaction with distinct E2F factors (D'Souza et al., 2001; Paramio et al., 2000). However, little is known about the function of this family during normal skin development and epidermal homeostasis *in vivo*. Transgenic mice expressing the viral oncoprotein HPV-E7, which binds and inactivates retinoblastoma family members, in epidermal cells develop epithelial cancer (Arbeit et al., 1994; Griep et al., 1993; Herber et al., 1996). In addition, *ink4<sup>Δ2,3</sup>;p21<sup>waf</sup>* doubly-deficient mice display severe alterations in epidermal differentiation (Paramio et al., 2001b). However, these studies have only addressed the function of pRb family members in an indirect manner. More recently, Balsitis et al. (Balsitis et al., 2003) have shown that *Rb* ablation in the epidermis mimics many of the features displayed by transgenic mice expressing E7 protein in the skin. However, concomitant expression of E7 in pRb-deficient skin aggravates this phenotype, indicating that E7 exerts *Rb*-independent activities (Balsitis et al., 2003), possibly by inactivating p107 and/or p130.

We have begun to dissect the specific and overlapping roles of pRb family in skin by analyzing mouse mutants deficient for one or more members. We recently demonstrated that the concomitant absence of p107 and p130 impairs terminal differentiation of skin in mice, whereas the onset of differentiation was unaltered (Ruiz et al., 2003). The embryonic lethality of *Rb*-null mutant mice precludes the phenotypic analysis of pRb in the epidermis, whose morphogenesis starts at 14.5 dpc (Byrne et al., 1994). Using a conditional knockout approach based on the *Cre/loxP* system (Sauer and Henderson, 1988) we have examined the role of pRb and p107 in epithelial proliferation and differentiation by targeted deletion of *Rb* in stratified epithelia using keratin *K14*-driven Cre recombinase (*K14cre*) (Jonkers et al., 2001).

Our results demonstrate that pRb plays an essential role as a regulator of epidermal homeostasis. Importantly, the consequences of *Rb* loss differ dramatically between the closely related follicular and interfollicular epidermis. Finally, Rb and p107 have shared roles in the epidermis in which p107 can compensate for loss of Rb function in differentiation and proliferation.

## Materials and methods

### Mice and histological procedures

*Rb<sup>F19/F19</sup>*, *K14cre*, ROSA-26 reporter and *p107<sup>-/-</sup>* animals were genotyped as described (Jonkers et al., 2001; Marino et al., 2000).

Skin samples were fixed in 10% buffered formalin or in 70% ethanol, and embedded in paraffin wax. Sections (5 μm) were stained with Haematoxylin/Eosin or processed for immunohistochemistry. Cryosections (8 μm) were prepared from skin samples embedded in OCT medium. For BrdU studies, mice were injected (0.1 mg/g weight) 1 hour prior to sacrifice (Santos et al., 2002).

### Antibodies and immunofluorescence

Immunofluorescence was performed using standard protocols on deparaffinized sections using antibodies against K10 (K8.60 mAb, 1/500 dilution, Sigma), K5 and K6 (both at 1/500 dilution, rabbit polyclonal antibody, Covance). BrdU incorporation was monitored in formalin-fixed sections as described (Santos et al., 2002). Horseradish-peroxidase, Texas-Red- and FITC-conjugated secondary antibodies were purchased from Jackson and used 1/4000, 1/500 and 1/50 dilution, respectively. Apoptosis was monitored using Apoptag kit (Oncor). Peroxidase was visualized using DAB kit (Vector). Control slides were obtained by replacing the primary antibody with PBS (data not shown). For *lacZ* staining, adult mouse tissues were briefly fixed in 0.2% PFA and equilibrated in 30% sucrose/PBS at 4°C and embedded in OCT compound. Cryosections (10 μm) were stained for β-galactosidase activity as described (Jonkers et al., 2001).

### Primary keratinocytes cultures

Primary keratinocytes were obtained from newborn mice skin and cultured in EMEM medium supplemented with 4% Chelex-treated fetal bovine serum (Biowhitaker), 0.05 mM of CaCl<sub>2</sub>, EGF (10 ng/ml, Serono) and antibiotics. Differentiation was induced by adding Ca<sup>2+</sup> up to 1.2 mM in the culture medium. BrdU was added to the medium (10 μm) and cells were incubated for 1 hour after which cells were fixed and processed for immunohistochemistry. Three independent experiments were carried out and at least 1000 cells were scored on each. Keratinocyte growth analyses were performed by plating 10<sup>5</sup> cells and counting cell numbers daily. Adenoviral infections were performed by incubating primary keratinocytes with Adeno-cre or Adeno-GFP supernatants for 2 hours in the presence of Polybrene in mixed DMEM/Optimem (50%) and 2.5% FCS. Afterwards, the medium was changed to standard low Ca<sup>2+</sup> medium.

### Western blot analysis

Primary keratinocytes were lysed in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 20 mM NaF, 1 μg/ml aprotinin and leupeptin, 1 mM disodium pyrophosphate and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Total protein (25 μg) was used for SDS-PAGE, transferred to nitrocellulose (Amersham) and probed with antibodies against pRb (PharMingen), p107 and p130 (Santa Cruz) all used at 1/1000 dilution. Actin (Santa Cruz) was used for normalization. Detection was performed using ECL (Pierce).

### Luciferase assays

Plasmid pSV40-Renilla was obtained from Promega. pE2F-Luc was a generous gift from Dr X. Lu. Primary mouse keratinocytes were transfected using Superfect (Quiagen) with pSV-Renilla (0.1 μg) and pE2F-Luc (2.4 μg). Lysates were prepared and analyzed with the Dual Luciferase Reporter Assay system (Promega). Relative luciferase expression was determined as the ratio of firefly to *Renilla* luciferase activity. Transfections were performed in triplicate, and the mean and standard error were calculated for each condition. Two independent transfection experiments were performed and luciferase activity was normalized to the values obtained with control, *Rb<sup>F19/F19</sup>*, cells cultured in low calcium.

### Label-retaining cell analysis

Ten-day-old pups were injected with BrdU (20 μl of a 12.5 mg/ml dilution in NaCl 0.9%) every 12 hours for a total of four injections. Skin sections were collected at 30 and 75 days after the last injection, and BrdU incorporation was measured as percentage of hair follicles

containing positive cells. Four different animals of  $Rb^{F19/F19}$  and  $Rb^{F19/F19};K14cre$  were used to count at least 100 follicles in each time point.

### Dye penetration assay

The epidermal permeability barrier function was monitored as described previously (Hardman et al., 1998). Briefly, unfixed and freshly isolated new born mice were rinsed in PBS then immersed in 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) reaction mix at pH 4.5 [100 mM NaPO<sub>4</sub>, 1.3 mM MgCl<sub>2</sub>, 3 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 3 mM K<sub>4</sub>Fe(CN)<sub>6</sub> and 1 mg/ml X-gal] and incubated at room temperature overnight.

## Results

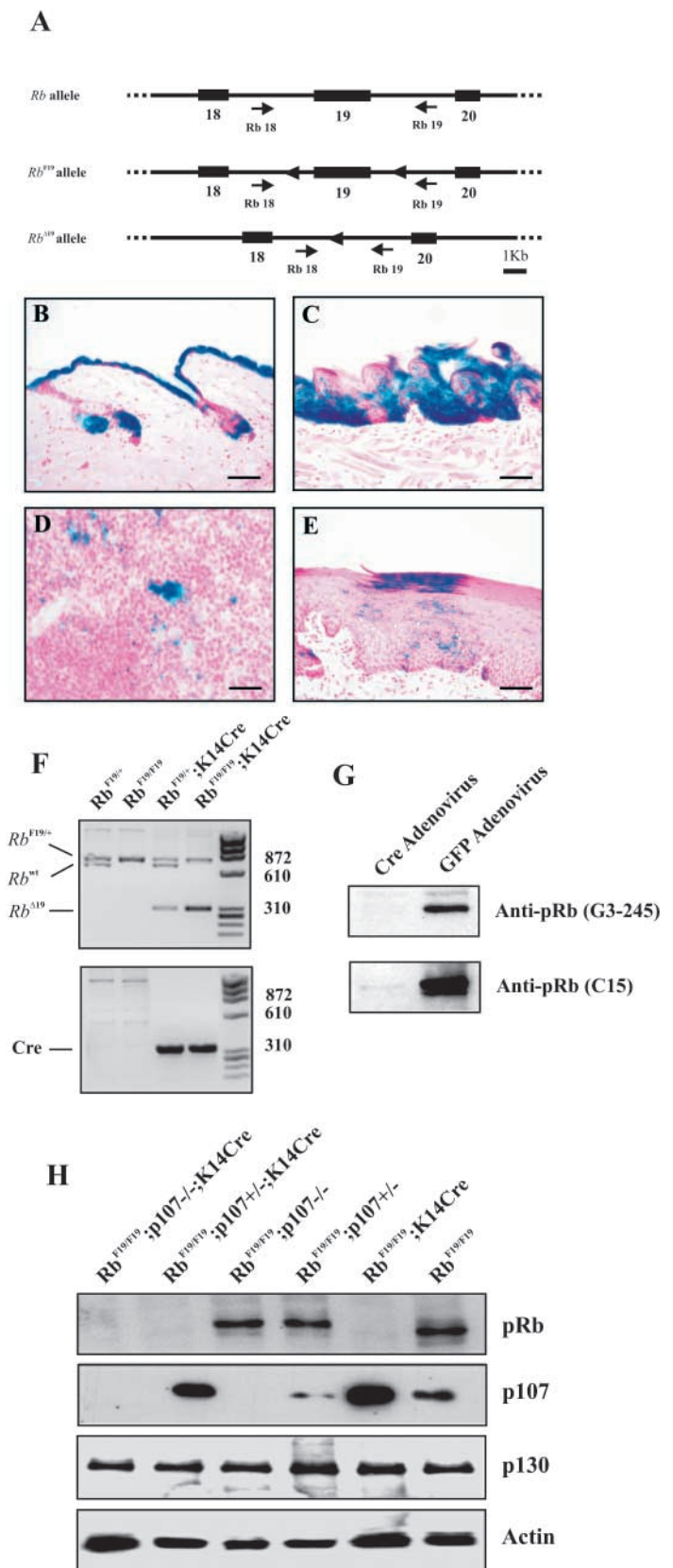
### Epithelial-specific inactivation of *Rb* leads to upregulation of p107

Mice with a conditional mutant *Rb* allele harboring *loxP* sequences surrounding exon 19 of the *Rb* gene (Fig. 1A) have been described (Marino et al., 2000; Vooijs et al., 2002a). The conditional deletion of exon 19 of *Rb* ( $Rb^{F19}$ ; Fig. 1A) results in a null allele ( $Rb^{\Delta 19}$ ) that phenocopies the embryonic lethality seen in *Rb*-knockout embryos and predisposes mice to tumor formation (Vooijs et al., 2002a; Vooijs et al., 2002b). To achieve epithelial specific inactivation of *Rb* we expressed Cre recombinase under the control of the keratin 14 promoter (Jonkers et al., 2001), which is normally expressed in basal cells of stratified epithelia (Byrne et al., 1994). To assess the cell type-specific recombination imposed by *K14cre* we crossed *K14cre* mice with *Rosa26* reporter (*R26R*) mice to obtain double transgenic *K14cre:R26R* animals (Soriano, 1999). X-gal staining demonstrated Cre activity throughout the follicular and interfollicular epidermis, and the epithelial linings of the tongue, palate, stomach, esophagus and thymic medullar epithelial cells (Fig. 1B-E, data not shown). The expression pattern of *K14cre* resembles the normal expression pattern of K14 (Byrne et al., 1994; Jonkers et al., 2001). No staining was observed in tissues that do not express K14 (data not shown).

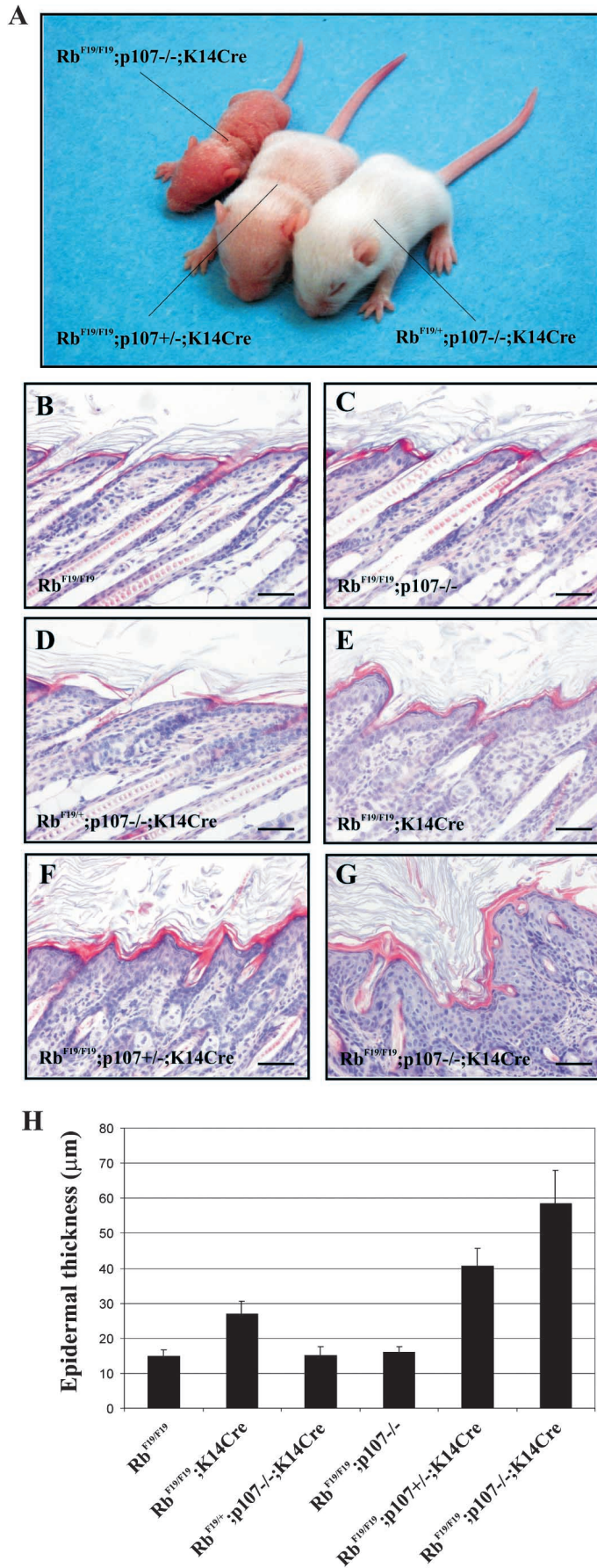
To achieve epithelial-specific deletion and inactivation of *Rb*, we mated  $Rb^{F19/F19}$  mice with *K14cre* transgenic mice. Cre-mediated *Rb* deletion in the offspring was readily detectable by PCR analyses on tail tip DNA and was only seen in *K14cre* transgenic mice (Fig. 1F).

To determine whether *K14cre* expression in the skin of  $Rb^{F19/F19}$  mice resulted in pRb loss, we analyzed total cell

lysates from primary keratinocytes derived from neonate skin by immunoblotting (Fig. 1H). Whereas keratinocytes derived from  $Rb^{F19/F19}$  mice expressed normal levels of pRb,



**Fig. 1.** Keratinocyte-specific Cre expression leads to pRb ablation in vivo. (A) Schematic representation of the wild-type, the floxed (F19) and the inactivated *Rb* allele ( $\Delta 19$ ). Exons are indicated as numbered boxes and *loxP* sites as triangles. Rb18 and Rb19 indicate the primers used to characterize the different *Rb* alleles. (B-E)  $\beta$ -Galactosidase staining (blue) identifies Cre activity in X-Gal stained tissue sections from epithelia of double transgenic *R26R:K14cre* animals in epidermis (B), tongue (C), thymic epithelium (D) and hard palate (E). (F) Cre-mediated recombination detected by PCR analysis on tails of  $Rb^{F19/F19};K14cre$  mice. (G) Immunoblot analysis for pRb in  $Rb^{F19/F19}$  keratinocytes 48 hours post-infection with Ad-GFP or Cre-coding adenovirus. (H) Immunoblot analysis for pRb family members on primary keratinocytes derived from newborn mice with the indicated genotypes. Scale bars: 150  $\mu$ m.



$Rb^{F19/F19};K14cre$ , keratinocytes showed complete loss of pRb (Fig. 1H). To substantiate this observation, we isolated  $Rb^{F19/F19}$  keratinocytes and infected them with adenoviruses encoding Cre or GFP (Akagi et al., 1997). Lysates from Cre- but not GFP-infected  $Rb^{F19/F19}$  cells showed complete absence of pRb protein within 48 hours of infection (Fig. 1G). Antibodies recognizing either the C- or the N-terminal of pRb failed to detect any protein, indicating that the pRb $^{\Delta 19}$  protein or mRNA is very unstable in cultured cells. We did not detect any Cre-mediated deletion in parallel cultured dermal fibroblasts of  $Rb^{F19/F19};K14cre$  mice (not shown), consistent with the absence of dermal staining in  $K14cre:R26R$  mice.

Lysates from  $Rb$  deficient keratinocytes showed a strong increase in p107 levels (Fig. 1H), similar to that seen pRb-null fibroblasts (Dannenberget al., 2000; Hurford et al., 1997; Sage et al., 2003). By contrast, in p107-null keratinocytes, we detected no increase in pRb protein levels (Fig. 1H). The upregulation of p107 in pRb-deficient keratinocytes suggested that p107 might functionally compensate for  $Rb$  loss. To address this aspect, we generated  $Rb^{F19/F19};K14cre$  mice lacking one or both alleles of  $p107$  (i.e.  $p107^{+/-}$  and  $p107^{-/-}$ ). As expected, neither pRb nor p107 protein could be detected in  $Rb^{F19/F19};p107^{-/-};K14cre$  keratinocytes. We observed no changes in p130 expression among the different genotypes.

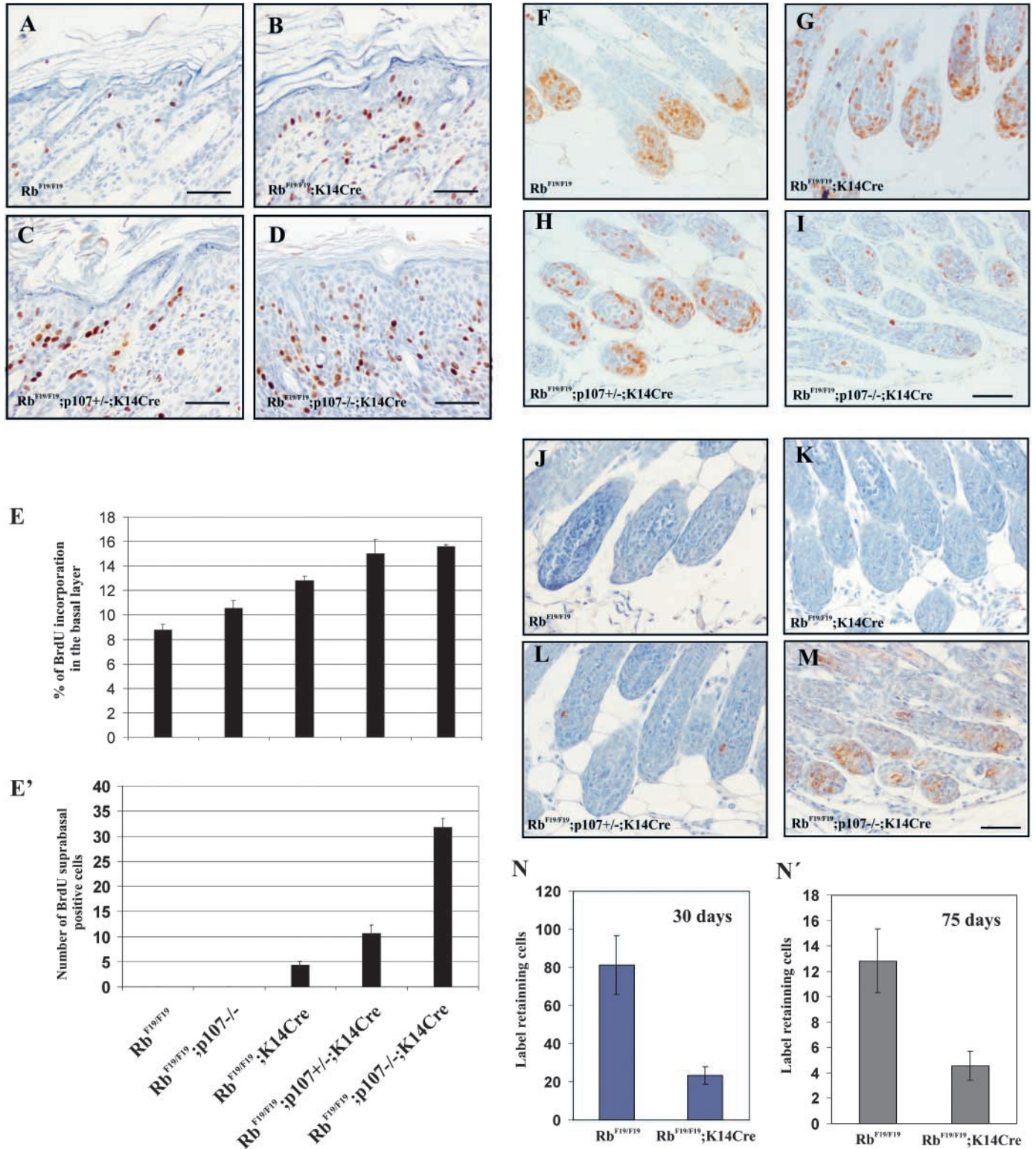
#### Phenotypic consequences of $Rb$ and/or $p107$ loss in the skin

$Rb^{F19/F19};K14cre$  mice were born at the expected Mendelian ratio and were indistinguishable from  $Rb^{F19/+}$  littermates in terms of external appearance until postnatal day 8 (P8), when  $Rb^{F19/F19};K14cre$  start showing a slight reduction in pelage hair and scaling (data not shown).  $Rb^{F19/F19};K14cre$  mice showed no detectable pathology, and served as controls (see below). This indicates that, in our experimental settings, the forced expression of Cre by itself does not contribute to any detectable phenotype (Loonstra et al., 2001).

The external appearance of the  $Rb^{F19/F19};K14cre$  mice was aggravated with concurrent loss of one or both  $p107$  alleles. Specifically,  $Rb^{F19/F19};p107^{+/-};K14cre$  mice showed a clear reduction in pelage hair and started developing an obvious scaling, whereas  $Rb^{F19/F19};p107^{-/-};K14cre$  showed essentially no hair, severe growth retardation and died between postnatal day 8 (P8) and P11 of an unknown cause (Fig. 2A). Importantly, whereas  $Rb^{F19/F19};K14cre$  mice showed a  $p107$  copy-number dependent aggravation of the

#### Fig. 2. Consequences of epidermal $Rb$ ablation in vivo.

(A) External appearance of P8 mice with epidermal-specific  $Rb$  loss. (B-G) Haematoxylin-Eosin (H/E) stained skin sections from  $Rb^{F19/F19}$  (B),  $Rb^{F19/F19};p107^{-/-}$  (C),  $Rb^{F19/+};p107^{-/-};K14cre$  (D),  $Rb^{F19/F19};K14cre$  (E),  $Rb^{F19/F19};p107^{+/-};K14cre$  (F) and  $Rb^{F19/F19};p107^{-/-};K14cre$  (G) mice at P8. There are no alterations in mice bearing one functional copy of  $Rb$  and the hyperplasia and hyperkeratosis promoted by epidermal pRb loss is evident, which becomes more progressive with the concomitant loss of p107. (H) Quantitative analysis of epidermal hyperplasia measured by epidermal thickness (in  $\mu m$ ) taken from three different age-matched mice of each genotype counting 2-4 sections in each (mean $\pm$ s.d.). Scale bars: 50  $\mu m$ .



**Fig. 3.** Proliferation defects in epidermal *Rb*-deficient mice. (A-D) Representative sections showing BrdU incorporation in the epidermis of  $Rb^{F19/F19}$  (A),  $Rb^{F19/F19};K14Cre$  (B),  $Rb^{F19/F19};p107^{+/-};K14Cre$  (C) and  $Rb^{F19/F19};p107^{-/-};K14Cre$  (D). (E-E') Quantitative analysis of the percentage of BrdU-positive nuclei per mm of epidermis in the basal layer (E) and number of BrdU-positive nuclei per mm in suprabasal layer (E') of mice with the indicated genotypes. Three 10-day-old mice of each genotype were analyzed, scoring 2-4 sections in each (mean $\pm$ s.d.). (F-I) Representative sections showing BrdU incorporation in hair follicles of 10 days old mice of each genotype. Scale bars: 100  $\mu$ m. (J-M) Apoptosis detection (TUNEL) in hair follicles of 10-day-old mice of each genotype. Scale bars: 100  $\mu$ m. (N,N') Label-retaining population at 30 (N) and 75 (N') days after BrdU labeling in mice of the quoted genotypes. Data in N and N' come from the study of five different mice analyzing 2-4 independent sections from each animal (mean $\pm$ s.d.).

external phenotype, a single wild-type *Rb* allele in *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup>;*K14cre* mice was sufficient to suppress this phenotype as these animals appear healthy, fertile and indistinguishable from wild type (Fig. 2A).

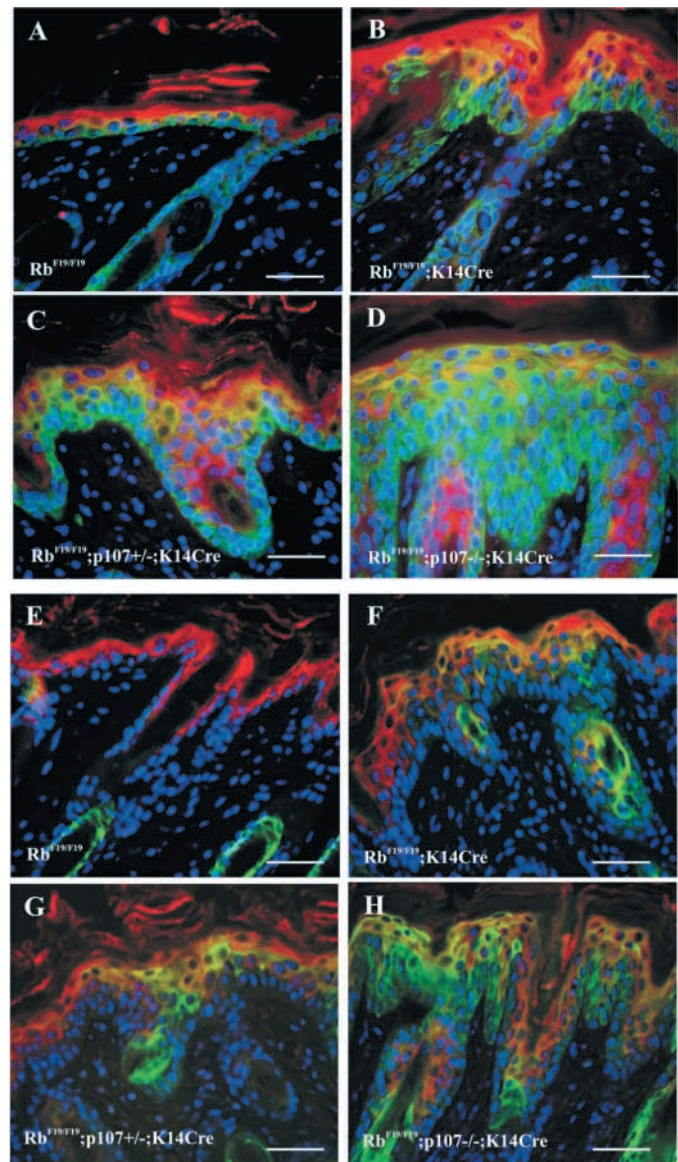
Histological examination of dorsal skin sections from *Rb*<sup>F19/F19</sup>;*K14cre* at P10 revealed a thickened epidermis characterized by mild hyperplasia, hyperkeratosis and increased cellularity in the dermis (Fig. 2E). The progressive loss of one or both copies of *p107* (Fig. 2F,G) aggravated this phenotype in a copy-number-dependent manner. This was further substantiated in a quantitative manner by measuring epidermal thickness of dorsal skin in mice of the different genotypes (Fig. 2H). *Rb*<sup>F19/F19</sup>, *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup> and *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup>;*K14cre* skins are indistinguishable from those of wild-type animals and were used as controls throughout these studies (Fig. 2B-D, respectively).

No obvious histological abnormalities were observed in tongue, palate, stomach or esophagus from *Rb*<sup>F19/F19</sup>;*K14cre* and *Rb*<sup>F19/F19</sup>;*p107*<sup>+/-</sup>;*K14cre* adult animals; hence, we focused our analysis on the skin.

### Proliferation defects in pRb-deficient skin

The increase in epidermal thickness and the hyperkeratosis suggest a defect in the regulation of proliferation and/or differentiation. To address this, we monitored BrdU incorporation at P10 in the basal layer of control mice, and *Rb*<sup>F19/F19</sup>;*K14cre*, *Rb*<sup>F19/F19</sup>;*p107*<sup>+/-</sup>;*K14cre* and *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup>;*K14cre* littermates (Fig. 3A-D). We observed a progressive increase in BrdU incorporation in the basal layer of *Rb*<sup>F19/F19</sup>, *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup>, *Rb*<sup>F19/F19</sup>;*K14cre* and *Rb*<sup>F19/F19</sup>;*p107*<sup>+/-</sup>;*K14cre* (Fig. 3E). Remarkably, we found no significant increase in BrdU incorporation between *Rb*<sup>F19/F19</sup>;*p107*<sup>+/-</sup>;*K14cre* (15.0±1.1) and *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup>;*K14cre* (15.6±0.2) (Fig. 3E). This was unexpected because the epidermal thickness significantly differed between these genotypes (Fig. 2H). A possible explanation for this observation could be the continued proliferation of pRb/p107-deficient suprabasal cells that normally arrest and undergo terminal differentiation (Fig. 3D). To test this hypothesis, we also determined the number of BrdU-positive suprabasal cells in each genotype. A significant increase in the number of proliferating cells dependent on the number of *p107* alleles was observed (Fig. 3E'). These results suggest that *p107*, in a dose-dependent manner, plays a crucial role in regulating the cell cycle exit in the absence of *Rb* in vivo but only plays a modest role in controlling basal cell proliferation in wild-type cells.

*Rb*-loss leads to apoptosis in the lens, central nervous system (CNS) and peripheral nervous system (PNS) (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). However, specific deletion of *Rb* by Cre/*loxP* technology revealed that apoptosis is not in all instances a cell-autonomous feature (Ferguson et al., 2002; MacPherson et al., 2003; de Bruin et al., 2003). Therefore, we analyzed whether loss of pRb in the epidermis leads to apoptosis. Whereas in the interfollicular epidermis the absence of pRb or pRb/p107 did not result in increased apoptosis (data not shown) we observed massive apoptosis in the hair follicles of *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup>;*K14cre* mice (Fig. 3J-M) with a concomitant reduction in BrdU incorporation (Fig. 3F-I). These alterations were not observed in any of the other genotypes (Fig. 3F-M) and probably account for the absence of hair pelage in these animals.



**Fig. 4.** Altered epidermal differentiation in *Rb*-deficient skin. (A-D) Double-immunofluorescence on P10 skin sections showing K5 (green) and K10 (red) or (E-H) K6 (green) and K10 (red) of *Rb*<sup>F19/F19</sup> (A,E), *Rb*<sup>F19/F19</sup>;*K14cre* (B,F), *Rb*<sup>F19/F19</sup>;*p107*<sup>+/-</sup>;*K14cre* (C,G) and *Rb*<sup>F19/F19</sup>;*p107*<sup>-/-</sup>;*K14cre* (D,H). Yellow staining indicates co-expression. DAPI (blue) was used to stain nuclei. Scale bars: 50  $\mu$ m.

### Alterations in epidermal stem cells in the absence of pRb

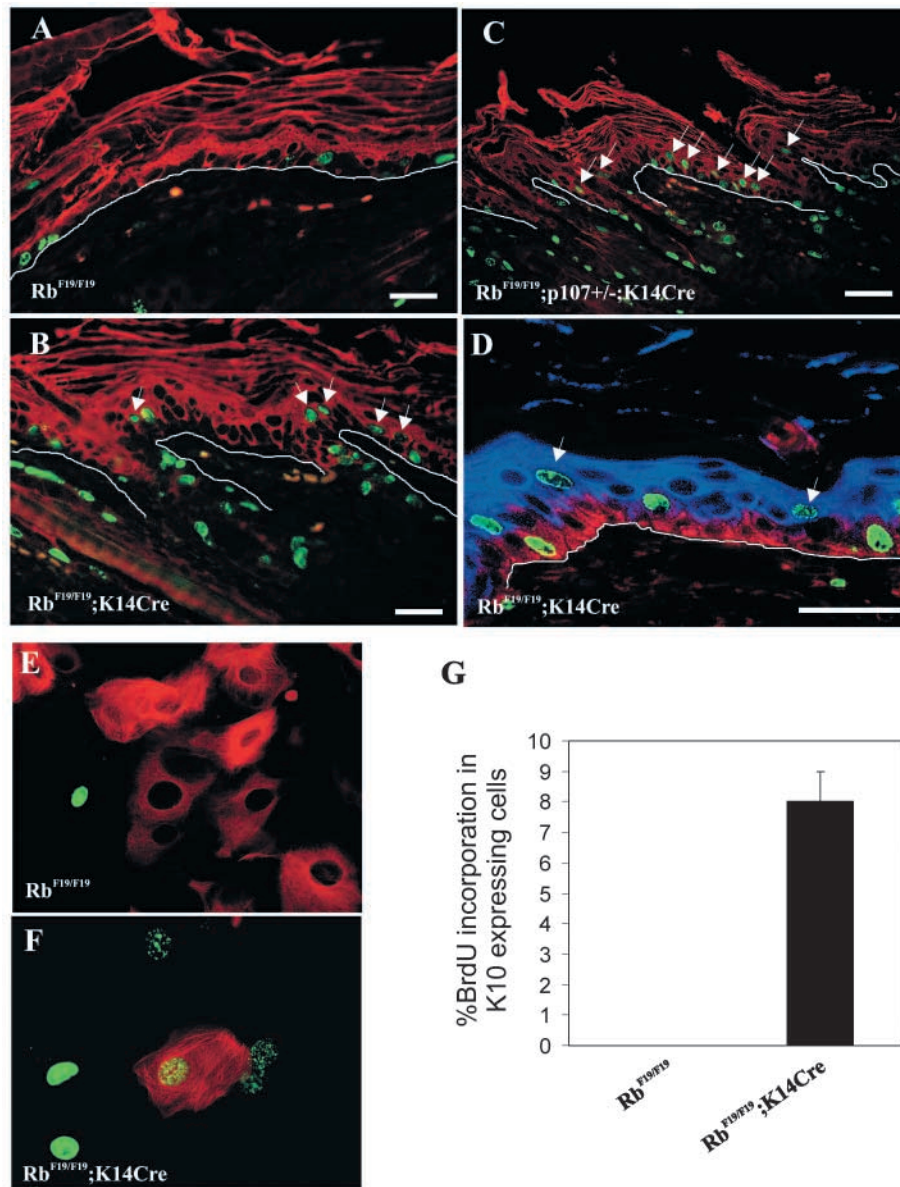
The transiently amplifying cells ensure the continuous cell renewal required in the epidermis as cells are shed from the surface. One prediction that stems from this is that increased cellular turnover caused by pRb loss would gradually deplete these progenitors as well as the epidermal stem cell compartment. Therefore, we determined whether the epidermal stem cell compartment was affected by pRb loss by using a label-retaining technique (Cotsarelis et al., 1990; Taylor et al., 2000). In *Rb*-deficient skin, we found a consistent reduction in the number of labeled cells after 30 (Fig. 3N) and 75 days (Fig. 3N') post BrdU administration, compared with control

littermates. This could indicate a reduction in stem cell number as a consequence of pRb loss.

### Differentiation defects in pRb-deficient skin

The abnormal proliferation of suprabasal cells in the skin of *Rb<sup>F19/F19</sup>;K14cre* prompted us to investigate whether there was any defect in differentiation. In normal epidermis, expression of keratin K5 is restricted to the basal layer, whereas expression of keratin K10 is found in the non-proliferative differentiated suprabasal layers (Fuchs and Green, 1980). Their expression patterns rarely overlap in normal mice (Fig. 4A). By contrast, in the *Rb<sup>F19/F19</sup>;K14cre* epidermis both the K5- and the K10-expressing cell layers are expanded, and cells co-expressing both K5 and K10 are regularly observed (Fig. 4B). This aberrant expression pattern is dramatically enhanced in *Rb<sup>F19/F19</sup>;p107<sup>+/-</sup>;K14cre* and *Rb<sup>F19/F19</sup>;p107<sup>-/-</sup>;K14cre* epidermis, respectively (Fig. 4C,D), where the K5-expressing cell layer was considerably expanded and few cells could be found that exclusively expressed K10 in the suprabasal layers. Importantly, most suprabasal cells co-expressed K10 and K5 and co-expression of these markers became more prominent with a reduction in p107 (compare Fig. 4B with D).

In epidermal hyperplasia, loss or downregulation of K10 is accompanied by the induction of keratin K6, a keratin normally expressed in hair follicles but not in the interfollicular epidermis (Takahashi et al., 1998; Weiss et al., 1984). We found that in *Rb<sup>F19/F19</sup>;K14cre* mice, patches of cells showed overlap of K6 and K10 expression throughout the hyperplastic epidermis (Fig. 4F). In *Rb<sup>F19/F19</sup>;p107<sup>+/-</sup>;K14cre* and *Rb<sup>F19/F19</sup>;p107<sup>-/-</sup>;K14cre* mice, there was a profound increase in ectopic K6 expression throughout the suprabasal layers accompanied by a decrease in K10-positive cells (Fig. 4G,H, respectively). By contrast, the expression of filaggrin and loricrin, two markers of terminal differentiation were comparable with wild-type mice (data not shown). Thus, despite the inappropriate proliferation and differentiation, pRb/p107-deficient keratinocytes appear to undergo some sort of terminal differentiation. We also examined whether the epidermal barrier was still intact in absence of *Rb* and/or p107 using a dye penetration assay (Hardman et al., 1998). We found that dye penetration was similar between pRb/p107-deficient



**Fig. 5.** Ectopic proliferation of differentiated *Rb*-deficient keratinocytes. (A-C,E,F) Double immunofluorescence against BrdU (green) and K10 (red) in the epidermis of adult *Rb<sup>F19/F19</sup>* (A), *Rb<sup>F19/F19</sup>;K14cre* (B) and *Rb<sup>F19/F19</sup>;p107<sup>+/-</sup>;K14cre* mice (C); and in *Rb<sup>F19/F19</sup>* (E) *Rb<sup>F19/F19</sup>;K14cre* (F) primary keratinocytes upon 24 hours of  $Ca^{2+}$ -induced differentiation. Arrows indicate BrdU incorporation in K10-expressing cells. (D) Triple immunofluorescence against K5 (red), K10 (blue) and BrdU (green) showing BrdU incorporation in cells expressing K10, but not K5 (arrows). (G) Summary of the BrdU incorporation in K10-positive primary keratinocytes of the indicated genotypes. Data are from the analysis of three independent experiments scoring at least 500 cells. White lines in A-C indicate the dermal epidermal junction. Scale bars: 50  $\mu$ m.

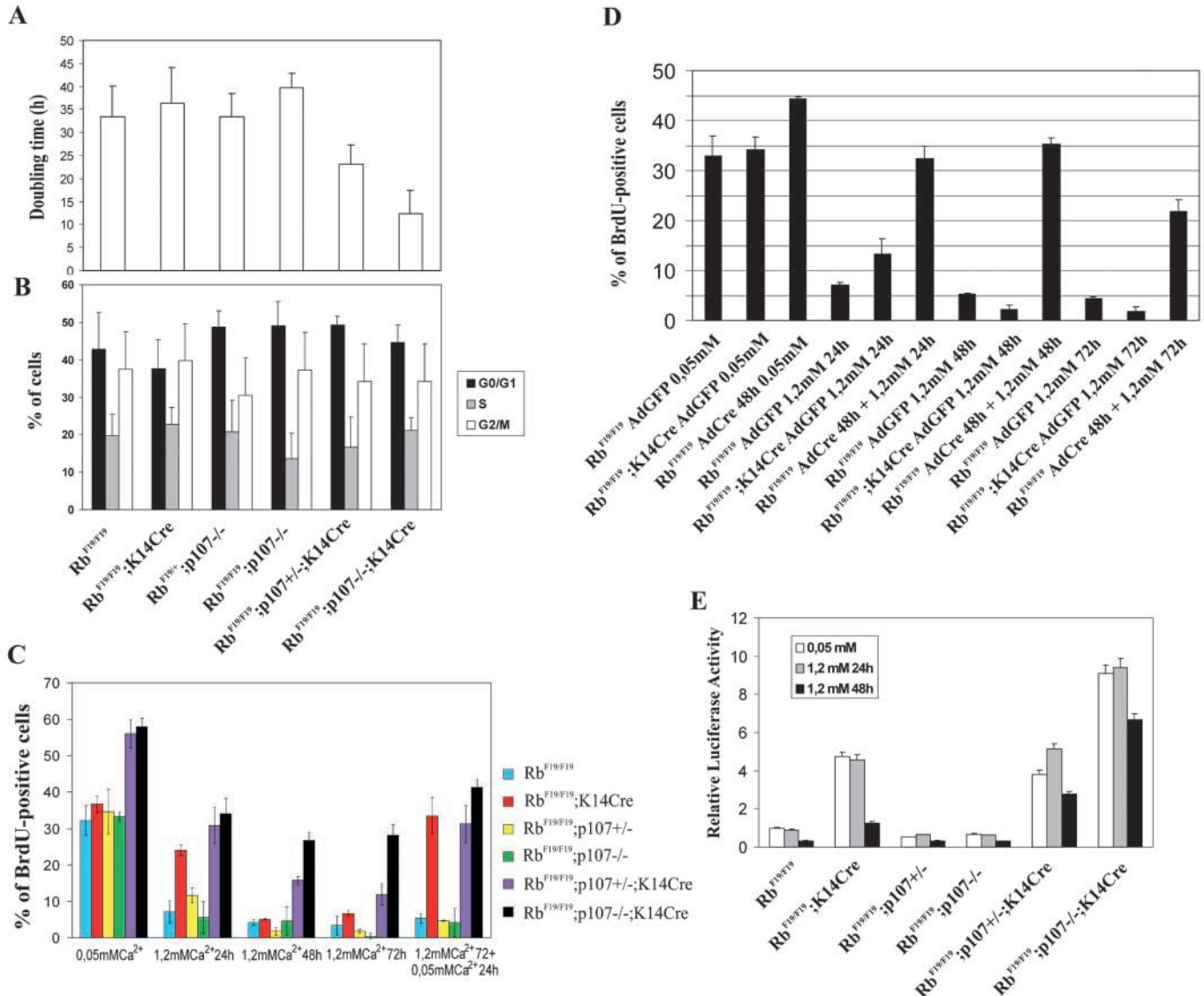
and wild-type epidermis, indicating that the observed defect in differentiation does not lead to a impairment of skin barrier.

### Differentiating epidermal *Rb*-deficient cells are ectopically dividing

The differentiation program in the epidermis takes place as the committed cells in the basal proliferative compartment arrest proliferation and move upwards towards the epidermal surface.

This differentiation is accompanied by changes in keratin expression. Therefore, we monitored BrdU incorporation in K10-expressing cells, the earliest marker of differentiation that appears *in vivo* or *in vitro*, when primary keratinocytes were induced to differentiate by calcium (see below). We found that *in vivo* as well as in cultured keratinocytes, *Rb*-deficient K10-expressing cells continued to proliferate (indicated by arrows in Fig. 5B,D,F,G) a phenomena infrequently seen in wild type keratinocytes (Fig. 5A,E,G). Because we found that in pRb-deficient skin both proliferation and differentiation of epidermal cells was affected (see also below), we investigated

whether dividing suprabasal K10+ cells had lost some of their basal characteristics (i.e. loss of K5 expression), as would be expected from a normal differentiating cell or whether: (1) pRb-deficient K5+ basal cells continued to divide and inappropriately turned on K10 expression; or (2) pRb-deficient basal cells had failed to turn off K5+ expression when becoming K10+. Using triple immunofluorescence staining against K5, K10 and BrdU, we found BrdU incorporation in some K10+, K5- cells (arrows in Fig. 5D). This indicates that proliferation and differentiation are uncoupled processes in pRb-deficient skin.



**Fig. 6.** Consequences of pRb and p107 loss in primary keratinocytes. (A) Doubling times of cultured primary keratinocytes showing a reduction in *Rb<sup>F19/F19</sup>;p107<sup>+/-</sup>;K14Cre* and *Rb<sup>F19/F19</sup>;p107<sup>-/-</sup>;K14Cre* with respect to wild-type and pRb-deficient cells. (B) FACS analysis of cell cycle profiles of asynchronous growing keratinocytes showing no significant differences among the different genotypes. Data are from the analysis of four independent experiments. In B, at least 10<sup>5</sup> cells were scored on each experiment (mean±s.d.). (C) Percentage of BrdU incorporation in keratinocytes growing under low (0.05 mM) and high (1.2 mM) Ca<sup>2+</sup> medium for the indicated times and re-stimulated with low Ca<sup>2+</sup> medium. (D) Percentage of BrdU incorporation in primary keratinocytes of the indicated genotypes after adeno cre or adeno GFP infection and before culture under the conditions indicated. Data come from the analysis of three independent experiments scoring at least 1000 cells on each (mean±s.d.). (E) Luciferase reporter activity of E2F of primary keratinocytes of the indicated genotypes cultured in low Ca<sup>2+</sup> or upon Ca<sup>2+</sup>-induced differentiation for 24 and 48 hours. Transfections were performed in triplicate, and the mean and standard error were calculated for each condition. Two independent transfection experiments were performed and luciferase activity was normalized to the values obtained with control, *Rb<sup>F19/F19</sup>*, cells in low Ca<sup>2+</sup>.



### pRb/p107-deficient keratinocytes exhibit increased proliferation

To further characterize the defects observed *in vivo* we monitored the proliferation of primary keratinocytes derived from these mice. We found a moderate decrease in the doubling time of *Rb<sup>F19/F19</sup>;p107<sup>+/-</sup>;K14<sup>cre</sup>* keratinocytes in comparison with the other genotypes (Fig. 6A) which became more pronounced with concurrent loss of p107. To investigate if this defect was due to alterations in any specific cell cycle phase, we performed flow cytometric analysis on asynchronous cultures. We did not find any significant alteration in G1, S or G2/M phases of the cell cycle in the absence of pRb and/or p107 (Fig. 6B). Moreover, we did not detect any change in a sub-G1 fraction indicative of apoptosis. This indicates that the increase in growth rate in pRb/p107-deficient keratinocytes might be due to an overall shortening of the cell cycle rather than an accelerated entry into S phase.

### Rb-deficient keratinocytes cannot maintain a differentiation-associated permanent growth arrest

The aberrant proliferation in the suprabasal layers of pRb-deficient epidermis, and the BrdU incorporation observed in K10-expressing cells *in vitro*, prompted us to investigate whether pRb-deficient keratinocytes still respond to stimuli that induce growth arrest. Cultured mouse keratinocytes respond to increases in Ca<sup>2+</sup> concentration in the medium (from 0.05 mM to 1.2 mM) by cell cycle arrest and expression of terminal differentiation markers (Hennings et al., 1980). We observed that *Rb*-deficient keratinocytes underwent a growth arrest similar to that observed in *Rb<sup>F19/F19</sup>* keratinocytes, albeit with a 24 hour delay in their response (Fig. 6C). On the contrary, a high fraction of the pRb;p107-null keratinocytes remained refractory to the Ca<sup>2+</sup>-induced growth arrest over a period of 72 hours. Importantly, a single wild-type *p107* allele in pRb-deficient keratinocytes was sufficient to suppress the growth rate of *Rb*-deficient keratinocytes within 72 hours.

To address if the Ca<sup>2+</sup>-induced growth arrest reflected a permanent arrest characteristic of postmitotic terminally differentiated keratinocytes *in vivo*, wild-type and pRb-deficient keratinocytes treated for 72 hours in high Ca<sup>2+</sup>, were re-stimulated by low Ca<sup>2+</sup> medium (0.05 mM) for 24 hours and assayed for BrdU incorporation. As expected, wild-type keratinocytes did not incorporate BrdU, demonstrating that Ca<sup>2+</sup>-induced differentiation is associated with a permanent cell cycle withdrawal. By contrast, pRb-deficient keratinocytes could completely overcome this growth arrest and be stimulated to divide to a similar extent as exponentially growing cells within 24 hours after removing Ca<sup>2+</sup> (Fig. 6C). In *Rb<sup>F19/F19</sup>;p107<sup>+/-</sup>;K14<sup>cre</sup>* keratinocytes, removal of Ca<sup>2+</sup> resulted in a threefold increase in the growth fraction; however, only a moderate increase in BrdU incorporation was seen in *Rb<sup>F19/F19</sup>;p107<sup>-/-</sup>;K14<sup>cre</sup>* keratinocytes.

It has been demonstrated that acute, but not permanent, loss of pRb in mouse embryo fibroblasts is sufficient to trigger cell cycle re-entry in cells grown under conditions which induce senescence or quiescence (Sage et al., 2003). To study if similar events take place during Ca<sup>2+</sup>-induced differentiation, we infected *Rb<sup>F19/F19</sup>* keratinocytes with cre-encoding adenovirus. Cre recombinase expression leads to a complete reduction in pRb protein levels within 48 hours after infection (Fig. 1G). Interestingly, in these cells we could not detect any

compensatory increase in p107 or p130 levels, as was seen in primary keratinocytes with constitutive pRb loss (data not shown). A modest increase in BrdU incorporation was observed by comparing *Rb<sup>F19/F19</sup>* keratinocytes 48 hours after Ad-cre infection with Ad-GFP infected *Rb<sup>F19/F19</sup>* or *Rb<sup>F19/F19</sup>;K14<sup>cre</sup>* primary keratinocytes (Fig. 6D). As demonstrated above, *Rb<sup>F19/F19</sup>* and *Rb<sup>F19/F19</sup>;K14<sup>cre</sup>* keratinocytes underwent a similar cell cycle arrest upon Ca<sup>2+</sup>-induced differentiation. By contrast, Ad-cre-infected *Rb<sup>F19/F19</sup>* keratinocytes were completely refractory to a calcium induced arrest within 48 or 72 hours. These results demonstrate that, similar to senescent or quiescent embryo fibroblasts (Sage et al., 2003), a substantial functional compensation by p107 is not immediately installed after acute loss of pRb.

### E2F activity is deregulated in Rb-deficient keratinocytes

During proliferation, growth arrest and differentiation, the activity of the E2F family of transcription factors is inhibited by interactions with pRb family members (Lipinski and Jacks, 1999). Because *Rb*-deficient keratinocytes were impaired in both the initiation and maintenance of a Ca<sup>2+</sup>-induced growth arrest, we investigated whether these defects were a consequence of deregulated E2F function. We measured E2F function in keratinocytes by transfection of an E2F-Luciferase reporter construct in wild type and pRb-deficient keratinocytes. In *Rb<sup>F19/F19</sup>;K14<sup>cre</sup>* keratinocytes E2F activity was increased fivefold over controls and even further increased in cells lacking both pRb and p107 (Fig. 6E). Upon Ca<sup>2+</sup> treatment, E2F activity is downregulated to wild-type levels in *Rb*-null keratinocytes closely following to the kinetics of growth arrest in these cells (Fig. 6C). However, in *Rb<sup>F19/F19</sup>;p107<sup>+/-</sup>;K14<sup>cre</sup>* and *Rb<sup>F19/F19</sup>;p107<sup>-/-</sup>;K14<sup>cre</sup>* keratinocytes E2F activity remained high up to 48 hours after the addition of Ca<sup>2+</sup> in accordance with their reduced sensitivity to Ca<sup>2+</sup>-induced differentiation (Fig. 6D,E).

## Discussion

Germline inactivation of *Rb* in mice leads to mid-gestational lethality that precludes the analysis of pRb deficiency at later stages of development and in the adult animal. The use of conditional knockout approaches based on the Cre/*loxP* technology may help to circumvent this problem *in vivo*. We and others have validated this approach in a variety of tissues *in vivo* (Marino et al., 2000; Vooijs et al., 2002b; Ferguson et al., 2002; MacPherson et al., 2003). While this work was in review, others have also reported conditional inactivation of *Rb* in the epidermis (Balsitis et al., 2003). In this model, the *Rb*-deficiency phenotype was further aggravated by co-expression of the HPV E7 protein, suggesting that pRb inactivation in K14-E7 transgenics was not complete and that other targets of E7 probably inactivation of p107 and/or p130 are involved (Balsitis et al., 2003). By analyzing the single and combined effects of skin-specific deletion of *Rb* and *p107*, we were able to further dissect the role of pRb family members in the epidermis. We found that *Rb* loss in the epidermis leads to hyperplasia and hyperkeratosis (Fig. 2) associated with defects in proliferation and differentiation (Figs 3, 4). Whereas the increased proliferation in the interfollicular epidermis leads to hyperplasia and inappropriate differentiation, loss of pRb in

conjunction with p107 in the hair follicles leads to profound apoptosis. This probably explains the hair loss seen in aged mice with *K14cre* induced *Rb* loss. Furthermore, apoptosis was enhanced in hair follicles lacking both *Rb* and p107. Transplantation of *Rb/p107*-deficient skins onto *nude* mice led to a further aggravation of the hyperplasia but no hair formed (data not shown). It has recently been demonstrated that the apoptosis seen in the CNS of pRb mutant embryos is non-cell autonomous (de Bruin et al., 2003). By contrast, our analysis shows that the apoptosis caused by loss of pRb in the hair follicles is cell autonomous. These results indicate remarkable differences in the consequences of pRb family loss in two closely related cell types: follicular and interfollicular keratinocytes.

Similar to pRb-deficient fibroblasts (Dannenbergh et al., 2000; Hurford et al., 1997; Sage et al., 2003), we detected increased p107 levels in pRb-null keratinocytes, probably because of the release of transcriptional repression of *p107* by pRb/E2F complexes (Zhu et al., 1995). This suggests that the increase in p107 protein levels could be a mechanism to compensate for loss of pRb function. We tested this possibility, and found that concurrent loss of *p107* in a *Rb*-null epidermis aggravated the 'pRb-deficiency' phenotype (Figs 2, 3). Moreover, compensation by p107 occurs in a dose-dependent manner indicating that p107 levels are limiting in *Rb*-deficient cells. It has been demonstrated that E2F transcription factors that normally bind pRb (i.e. E2F1, E2F2, E2F3) may bind p107 in *Rb*-deficient cells (Lee et al., 2002). Our results support this observation and further indicate that functional compensation in *Rb*-deficient keratinocytes by p107 crucially depends on the absolute levels of p107 protein.

It is noteworthy to mention that one functional copy of *Rb* is sufficient to rescue defects associated with p107 deficiency, as *Rb<sup>F19/+</sup>;p107<sup>-/-</sup>;K14cre* keratinocytes behave normally in vitro and in vivo. This may be, in part, explained by the presence of functional p130 that could compensate for both pRb and p107 loss. This is in line with our recent finding that p107 and p130 perform crucial overlapping functions in the epidermis in vivo (Ruiz et al., 2003). The generation of mice lacking all pocket proteins in the epidermis will be needed to define the capacity of each of these family members to substitute for the others.

In the epidermis, the proliferative basal layer and differentiating suprabasal layers are spatially separated and terminal cell cycle arrest precedes the onset of differentiation. Our results indicate that, in the absence of *Rb*, differentiation and proliferation are uncoupled processes in vivo, since pRb-deficient cells actively proliferate while initiating the expression of differentiation markers (Fig. 5, Fig. 6C,D). Similarly, the analysis of pRb-deficiency in telencephalon also revealed that mitotic arrest is not required to initiate neural differentiation (Ferguson et al., 2002).

Epidermal homeostasis requires continuous self-renewal of keratinocytes by transit amplifying cells that are derived from stem cells (Watt, 2001; Alonson and Fuchs, 2003). Most adult stem cells divide infrequently and give rise to committed progenitors (Watt, 2001; Alonson and Fuchs, 2003). This feature permits the quantitation of stem cell niches by identification of label-retaining cells (Cotsarelis et al., 1990). Using this technique, we found a decrease in the label retaining cell population in *Rb<sup>F19/F19</sup>;K14cre* compared with wild-type

mice. This might either indicate a depletion of epidermal stem cells or an increased rate of cell division of epidermal stem cells in the absence of pRb, similar to that seen in basal layer keratinocytes. Currently we cannot distinguish between these possibilities.

Although epidermal differentiation ensues in *Rb*-deficient skin, it is highly perturbed, as demonstrated by the ectopic expression of K6, suprabasal expression of K5 and a reduction in the number of K10-expressing cells (Fig. 4). These defects became more pronounced in the absence of p107, which again indicates that p107 can partially compensate for *Rb*-loss in the epidermis. We detected no changes in the expression of terminal differentiation markers filaggrin or loricrin in pRb-deficient epidermis (data not shown). By contrast, in the epidermis of mice lacking p107 and p130 where the initial steps of differentiation are unaffected a clear defect in terminal differentiation is seen (Ruiz et al., 2003). These functional analyses fit nicely with the overlapping and unique expressing patterns and functions proposed for pRb family members in skin (Paramio et al., 1998).

Our results demonstrate a strong functional overlap between pRb and p107 genes in epidermis and illustrate a dose-dependent effect of p107 in vivo in the context of a pRb deficiency. In addition, these results illuminate functional differences between pRb and p107 in the ability to suppress epidermal hyperplasia and regulate differentiation. To date, we are not able to discriminate if proliferation or differentiation defects are due to the E2F release or to a failure in the active transcriptional repression mediated by pRb/E2F complexes. In addition interactions with transcription factors, different from E2F, might contribute to the resultant phenotype.

The mechanism whereby pRb family members directly or indirectly regulate the onset of differentiation is unknown at present. It is well established that *Rb* family members regulate the expression of differentiation markers through direct interaction with transcription factors, such as MyoD in muscle (Novitsch, 1996) and C/EBP $\beta$  in lung fibroblasts (Chen, 1996). Interestingly, C/EBP $\beta$  controls proliferation in keratinocytes and directly regulates the expression of K10 (Zhu, 1999; Maytin, 1999; Charles, 2001). However, we did not find any alterations in C/EBP $\beta$  activity during differentiation of *Rb*-deficient keratinocytes compared with control cells (not shown), suggesting that this transcription factor is not deregulated in *Rb*-deficient keratinocytes.

Among the differentiation alterations observed in absence of pRb, the reduced expression of K10 (Fig. 4) might be of a particular relevance. Our recent work has demonstrated that this keratin may impose a cell cycle arrest in keratinocytes in a pRb-dependent manner (Paramio et al., 1999; Paramio et al., 2001a; Santos et al., 2002). Here, we show that K10-expressing cells do not exhibit a proliferative arrest. Given that K10 induces a cell cycle arrest in vivo and suppresses tumor development (Santos et al., 2002), the possible loop between pRb and K10 merits further investigation.

The in vitro and in vivo analysis presented here support a dual role for pRb family members in keratinocyte differentiation. First, pRb is essential to initiate a growth arrest in response to differentiation signals. However, in the sustained absence of pRb, p107 is necessary and sufficient to initiate this proliferative arrest (Fig. 6C), probably owing to the increase in p107 levels that accompany permanent loss of pRb (Fig. 1H).

Second, pRb is necessary and sufficient for the maintenance of differentiation-induced postmitotic state in keratinocytes (Fig. 6C). A similar role for pRb and p107 in the maintenance of terminal differentiation of myocytes has also been reported (Schneider et al., 1994). It is currently not understood whether pRb has a unique role in maintaining terminal postmitotic state or whether p107 levels are simply not high enough to maintain a growth arrest. Sage and colleagues recently reported similar findings in fibroblasts with conditionally inactivated pRb (Sage et al., 2003). Maintenance of the quiescent state is essential for normal development and differentiation. Perturbations in this cellular control lead to inappropriate proliferation and expansion of cell compartments that are at increased risk of acquiring cancer prone mutations. These data may help to explain why *Rb*, but not *p107*, is predominantly found mutated in human tumors.

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