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Of mice without pockets: mouse models to study the function of *Rb* family proteins

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1. ABSTRACT

Three negative regulators of cell cycle, the related proteins, pRB, p107 and p130, constitute the family of pocket proteins. pRB is a tumor suppressor which has drawn a lot of attention on its family of proteins, with the ensuing intense study of their biology. As a result we have a wealth of information on their biochemistry and biology, ranging from their regulation to their biochemical activities, and the effects of their absence or overexpression on cells. Despite this, many questions remain unsolved. In recent years, analysis of genetically-modified mouse strains has provided interesting data regarding the physiological and pathophysiological roles of these three proteins. Specifically, germ-line and conditional knockout strains for one or more than one of the members of the family have revealed as powerful tools in this regard. Here we review the mouse models available for studying these cell cycle regulators and how data generated by these approaches have sometimes challenged previous thoughts about the pocket proteins biology.

2. INTRODUCTION

The retinoblastoma susceptibility gene (*Rb*) was cloned 30 years ago (1,2). *Rb* was soon shown to be mutated in an unexpectedly high number of cancers (3,4,5), and became a paradigmatical tumor suppressor gene. Two other proteins, p130 and p107, were identified little thereafter based on sequence homology with the product of *Rb* (pRB) and on their biochemical properties (6,7,8,9). Together, pRB, p107 and p130 are usually called pocket proteins because they share a domain (the pocket), that binds E2F and viral oncoproteins.

Several different biochemical functions have been ascribed to these proteins, but it is unclear which of these is relevant in a physiological setting. Moreover, several experiments have suggested that pocket proteins are redundant in some cells, but not in others. We are only beginning to understand the extent of this redundancy and its molecular basis. Last but not least, while *Rb* is clearly tumor suppressor, it is not so clear what is the status of

p107 and *p130* in this respect. These proteins have shown antiproliferative activities *in vitro*, and can compensate for the absence of pRB in some settings, including inhibition of tumor development. Nevertheless, whether they have tumor suppressor activity on their own awaits further investigation. Genetically modified animals, especially knockout mice, are helping us to answer these questions and should be instrumental in unravelling the biology and pathophysiology of pocket proteins. We will review here the knowledge we have gained on the biology of pocket proteins using genetically modified mice, with special focus on information gathered on tumor suppressor functions of the *Rb* family members.

3. BIOCHEMISTRY OF THE POCKET PROTEINS

We will first discuss some of the biochemical activities that have been attributed to the pocket proteins. For a thorough account of this specific issue, several excellent reviews have recently been published (10,11,12). As stated above, pocket proteins have been credited with several biochemical actions. Prominent among them is their ability to bind and repress E2F transcription factors. This family is composed of at least 9 members, that can be classified as transcriptional activators (E2F1, E2F2 and E2F3a), and repressors (E2F3b, E2F4, E2F5, E2F6, E2F7 and the recently cloned E2F8)(13,14,15,16). They bind to DNA in a sequence-specific manner to control transcription of genes involved in cell cycle control, apoptosis, and DNA repair (17,13,18). pRB can bind E2F1, 2, 3a, 3b, 4 and 5, whereas p107 and p130 bind preferentially to E2F4 and E2F5 (10), although they have also been found bound to E2F1 (19,20,21). These interactions result in the inhibition of E2F-dependent transcription, and several different activities of pocket proteins have been credited with this inhibition. Pocket proteins can block the transactivating domain of the E2F they bind, and thus inhibit transcription (22,23). Moreover, pocket proteins can mediate active repression of transcription through recruitment of histone modifiers like histone deacetylases (24,25,26) and histone methylases (27,28). They can also inhibit assembly of transcription initiation complexes (29). Besides, pRB has been reported to interact with proteins involved in nucleosome remodelling (30,31).

The activities of pocket proteins are regulated by phosphorylation. Cyclin D/CDK4 and cyclin E/CDK2 complexes have long been implicated in pocket protein inactivation (for a review see (32)). pRB and p130 have been shown to be phosphorylated by both cyclin D/CDK4 and cyclin E/CDK2 (33,34,20,35). Elegant experiments have identified CDK-specific phosphorylated residues on both proteins, and for pRB the consequences of its sequential phosphorylation have been described in detail (36,32,37). On the contrary, only cyclin D/CDK4 seems to be able to phosphorylate p107 (38). Cyclin E/CDK2 overexpression does not override a p107-mediated growth arrest, and adenovirus infection of these proteins in quiescent cells do not result in overt endogenous p107 phosphorylation (33), suggesting that the regulation of this protein is different from the other pocket proteins. Adding to this intriguing difference, p107 is found bound to E2F4 in late G1 and S phase (39), although the complex formed does not sit on E2F-dependent promoters (40). At this stage of the cycle, CDK-containing complexes are highly active, what suggests that the phosphorylation of p107 does not disassemble p107 from E2F4 although it may displace p107-E2F4 from promoters. Alternatively, the p107 seen bound to E2F4 may be newly synthesized protein not yet phosphorylated by cyclin/CDK complexes.

Repression of E2F activity is assumed to be a very important function of pocket proteins (if not the only one that is physiologically relevant). However, there are several questions that can be asked about this activity. Which of the mechanisms of E2F repression is relevant in a physiological setting? How is this activity regulated, and in what situations do the pocket proteins exert their repressive activities? What genes are regulated by these proteins? Are all E2F-dependent genes equally regulated? What are the effects of the actions of pocket proteins on the biology of a cell and the physiology of an animal?

Cells derived from mice with inactivated genes for each pocket protein or a combination of them, have been instrumental in identifying what genes are regulated by each of these proteins. In a first approach, Hurford et al compared genes that are deregulated in quiescent $Rb^{-/-}$ and $p107^{-/-}$; $p130^{-/-}$ MEFs respect to wild type cells. They showed that genes that are important for G1/S transition are deregulated in mutant cells. Cyclin E and p107 expression were high in quiescent $Rb^{-/-}$ cells, whereas B-Myb, cdc2, E2F-1, TS, RRM2 and cyclin A2 were derepressed in $p107^{-/-}$; $p130^{-/-}$ cells (41). Later assays of this kind have looked at a wider range of genes by using microarray-based technology, and have found specific expression signatures in $Rb^{-/-}$ and $p107^{-/-}$; $p130^{-/-}$ cells. Importantly, these signatures are conserved in *Rb*-negative tumors (42).

p130 is the major pocket protein present in many different E2F-dependent promoters in quiescent cells, together with repressor E2Fs (40,43), whereas both p107 and p130 can be found on these promoters in G1 of proliferating cells (44). This suggests that p130 is especially important for regulation of cells in G0, and p107 also plays a role in proliferating cells. Late in G1 phase and coincident with the activation of cyclin/CDK complexes and the phosphorylation of pocket proteins, p107 and p130 disappear from the promoters, and activating E2Fs instead of repressors are detected. The surprising part of these results is that pRB is found in a very limited set of E2F-dependent genes in quiescent or proliferating cells, the most prominent of them being that of cyclin E (43,45). This, together with its ability to attract a histone methylase to promoters and thus induce heterochromatin formation, has led to the suggestion that it may be recruited to E2F-dependent promoters on special situations, like senescence or differentiation (see below). On the other hand and as we have already stated, expression analysis of cells in quiescence have identified a group of genes deregulated in *Rb*-negative cells, indicating that *Rb* does have a role in transcription regulation in quiescence.

In addition to its role in quiescence, it seems clear that pocket protein activity is also important for the senescent state. Senescence is a form of irreversible growth arrest that has been recognized as an important antioncogenic mechanism (see (46) and references therein). pRB is typically activated during senescence, and its enforced expression induces senescence in some cell types (47). In fibroblasts, DNA tumor virus oncoproteins that interfere with pRB family function can bypass senescence, and cells lacking all three pocket proteins fail to senesce in culture (48,49). Moreover, knock down of *Rb* in human cells can overcome at least some of the events related to senescence (50). In senescent cells pRB is kept in a hypophosphorylated and thus active state because of their high levels of p16, an inhibitor of the cyclin D/CDK4 complex. As a result, pRB is attracted to E2F-dependent genes, where it can be detected (as opposed to quiescent cells), and induces formation of heterochromatin in their promoter and permanent repression of their activity (50).

Although E2F binding and repression is the best known biochemical activity of *Rb*-related proteins, other functions have been attributed to these proteins, although it is unclear at the moment which of them, if any, is important in a physiological setting. Both p107 and p130 have been shown to inhibit CDK2-containing complexes in a manner similar to the Cip/Kip family of CDK inhibitors and through a cyclin binding motif similar to that found in these proteins (51,52,53). In fact, p107 is known to be bound to cyclin E/CDK2 and cyclin A/CDK2 complexes in S phase cells (54,55), although the significance of this interaction is unclear. Moreover, p130 is required to inhibit CDK2 activity and maintain quiescence in $p27^{-/-}$ fibroblasts and splenocytes (56,57) and p107 is important for CDK2 inhibition and cell cycle withdrawal in $p27^{-/-}$; $p21^{-/-}$ fibroblasts that express a stabilized form of cyclin A (58,56). Despite of these functions, neither p107 nor p130 are known to be important in CDK inhibition in normal cells, although this function could be important to maintain quiescence in particular cell types as proposed for angioblasts (59). Although pRB does not have a cyclin binding motif as p107 and p130 have, it can stabilize p27 directly by binding to the F box protein Skp2, inhibiting its interaction with p27 and preventing the degradation of the latter, resulting in inhibition of CDK2-containing complexes. This biochemical action is necessary for pRB to induce rapid cell cycle withdrawal when overexpressed in *Rb*-negative osteosarcoma cells (60). p107 has also been proposed to influence Skp2 activity through destabilization of the protein in rat cells, although the biochemical activity underlying this effect is unclear (61).

One of the earliest described biological effects of pRB overexpression was its ability to inhibit growth and induce the appearance of so called flat cells in *Rb*-negative cells. These cells do not proliferate, have a very characteristic morphology that gives them their name, and express a number of differentiation markers (35). The fact that pRB can induce them is usually taken as an indication that it can promote differentiation. Mutants of pRB that do not bind to E2F can induce flat cells, suggesting that their differentiation-promoting activity is independent of E2F repression (62). In a different setting pRB has been proposed to promote adipogenic differentiation, while p107 and p130 inhibit it, what correlates with pRB's ability to transactivate the adipogenic transcription factor CEBPalpha (63). pRB can also stimulate transactivation of NeuroD1 and the Nurr family of transcription factors to activate transcription of the POMC promoter in the differentiation of pituitary corticotroph cells (64,65). Another transcription factor that can be bound by pRB is the osteogenic transcription factor Cbfa1/Runx2. pRB can stimulate Cbfa1-dependent transcription, and this may contribute to its induction of the flat cell phenotype in osteosarcoma cells (66). The mechanism by which pRB can perform this transactivation of differentiation specific markers is unclear, but some recent developments may help to shed some light to this issue. pRB has been found on promoters of differentiation-induced genes, such as BRD2 and BRD8, where it interacts with the protein RBP2, transactivating these genes (67). Also, pRB has been shown to interact with the human homolog of de *C. elegans* gene Lin9 (hLin9), which codifies a chromatin-associated protein. hLin9 can cooperate with pRB in the induction of flat cells but not in the growth arrest of *Rb*-negative cells, and it enhances transactivation of differentiation-specific genes by pRB (68).

Several pieces of data suggest that pocket proteins may have a direct effect on DNA replication. Pocket proteins colocalize with replication origins of early S phase on primary cells (69), and RBF, the *Drosophila melanogaster Rb* homolog, has been shown to interact with the origin recognition complex (70,71). On the other hand, pRB overexpression arrests cells in S phase, when E2F activity is not limiting (72). Nevertheless, what the activity of pocket proteins in replication origins may be, and whether it has a physiological significance awaits further experiments.

pRB has also been described to suppress both RNA polymerase I- and RNA polymerase III-dependent transcription. Inhibition of RNA polymerase III seems to happen through pRB binding to TFIIIB, and by pRB occupancy of some Pol III-dependent promoters, whereas inhibition of RNA polymerase I is dependent on pRB binding to the polymerase I transcription factor UBF. These biochemical activities of pRB have been proposed to be important for pRB's inhibition of cell growth (increase in cell mass), rather than for its inhibition of cell proliferation (73).

A very interesting activity of pocket proteins that is being unveiled in the last few years is related to heterochromatin formation. We have already mentioned the ability of pRB to promote changes in histone methylation on the promoters of E2F-dependent genes, both in quiescence and senescence. More recently, pocket proteins have been proposed to be important in trimethylation of histone H4, a histone modification characteristic of centromeric and telomeric chromatin. Cells devoid of pocket proteins have lower levels of trimethylated H4 than wild-type cells, what results in lower DNA methylation and deregulated telomere length. This effect is global and not

limited to the promoters of E2F-dependent genes, suggesting that it is completely novel and independent of the previously characterized transcriptional effects of the family (74,75).

4. MOUSE MODELS: CONVENTIONAL AND ADVANCED

The *Rb* gene was the first of the family to be inactivated by genetic targeting in mice (76,77,78). *Rb*$^{-/-}$ mouse embryos die in utero between days 13 and 15 of gestation. The defects in these mice affect several cell types. Neurons in their central nervous system have an altered regulation of cell cycle, with inappropriate S-phase entry and ectopic mitosis. *Rb*$^{-/-}$ embryos also have a disorganized architecture, altered cell cycle regulation and enhanced apoptosis in the lens. However, the most apparent effect of the lack of pRB derives from defects in extraembryonic tissue. *Rb*$^{-/-}$ embryos have an abnormal placenta, with excessive proliferation of trophoblast cells and a severe disruption of its normal labyrinth architecture. This leads to a reduction in the transport function of the organ and hypoxia of the embryo, resulting in several secondary effects that include altered erythropoiesis and massive cell death in the central nervous system and causes the death of the embryo. In fact, *Rb*$^{-/-}$ embryos with a wild-type placenta survive until birth, and do not show evident neurological and erythroid abnormalities. However, rescued *Rb*$^{-/-}$ embryos die at birth, showing a severe skeletal muscle dysplasia that apparently results in an inability to breath (79).

The *Rb*$^{-/-}$ mice exemplify the difficulty of finding a mechanism of a phenotype of a knockout animal. Their placental defect was not found until ten years after the mouse was developed and initially described, despite the high number of scientific papers that used these animals or cells derived from them. As a consequence, many of the analyses of the phenotype of the mice and the epistatic experiments with other genes focused on its very obvious effects on neuronal apoptosis and erythropoiesis. Given that we know now that these effects are secondary to the placental defect, analysis of the placenta should be performed to evaluate the contribution of this organ to the phenotypes described in each situation.

Despite of the profound consequences of the lack of pRB in mouse embryos, what was really surprising about the *Rb*$^{-/-}$ phenotype when it was first reported was the fact that mice lacking such an important cell cycle regulator could reach day 14 of gestation. From the first moment it was suggested that one or the two pRB-related proteins (p107 and p130), could be compensating for the lack of pRB. In fact, quiescent *R*b-negative cells have elevated levels of p107 (41) (as do *p130*$^{-/-}$ cells (80)), and *p107-Rb* double mutant embryos die with the same phenotype as their *Rb*$^{-/-}$ counterparts but 2 days earlier (81). This suggests that the lack of p107 makes the placental phenotype more severe, although no analysis of this organ in these animals has been reported so far. Furthermore, when *Rb-p107-p130* triple knockout cells were generated they were shown to be completely refractory to the induction of quiescence by any means, and apparently unable to give rise to normal mouse embryos, what shows that the three pocket proteins collaborate to regulate G1 transition (48,49). Interestingly, the effects of the absence of pRB on the G1/S transition are different in models of chronic versus acute deletion of the *Rb* gene. Thus, primary mouse embryo fibroblasts (MEFs) derived from germ-line constitutive *Rb*$^{-/-}$ embryos have high levels of p107 and can be induced into quiescence. But if *Rb* is acutely inactivated by expression of Cre recombinase in quiescent MEFs derived from *Rb*$^{lox/lox}$ mice, they enter S phase with no sign of G1 control probably because the cells do not have time to adapt and upregulate p107 in a compensatory fashion. (82).

Perhaps not unexpectedly, mutation of each of the E2F family members E2F1, E2F2 and E2F3, rescue some but not all of the phenotypes of *Rb*$^{-/-}$ mutant embryos (83,84,85). Effects known now to be secondary to the placental phenotype, such as apoptosis in the CNS and altered erythropoiesis, and cell autonomous phenotypes, as S phase entry in neurons and apoptosis in the lens, are rescued by the lack of E2F1 (84). Mutation of E2F3 has a greater effect in rescuing the proliferation defects in *Rb*$^{-/-}$ embryos. While it can also inhibit the CNS apoptosis dependent on their placental phenotype, it is controversial whether it can reverse apoptosis in the lens and retinas of *Rb*$^{-/-}$ embryos (83,85). Loss of E2F2 rescues the proliferation but not the apoptosis defects in *Rb*$^{-/-}$ embryos (83). Unfortunately, no analysis has still been made of the placenta in any of the double mutants, and this precludes a thorough understanding of the relations between these genes. Significantly, the rescue of the *Rb*$^{-/-}$ phenotype by an E2F mutation is not complete. Both *Rb*$^{-/-}$*; E2F1*$^{-/-}$ and *Rb*$^{-/-}$*; E2F3*$^{-/-}$ embryos survive longer but die at around day 17 of gestation, with defects in erythropoiesis, skeletal muscle cells and lung (84,85). This shows that the actions of pRB are not mediated by a single E2F gene.

Inactivation of *Id2*, a gene that encodes an antagonist of the basic helix-loop-helix transcription factors, rescues the phenotype of *Rb*$^{-/-}$ embryos in at least the same degree as the E2F1 knockout (86). Again, a detailed analysis of the contribution of the placental defects to the phenotypes of the different mutant mice would be important in this setting. At any rate, this important and unexpected observation has led to a reevaluation of the relevance of Id2 as a target for pocket proteins, as Id2 binds to pocket proteins and is inhibited by them (87). Since *Id2* is also a target of the c-myc transcription factor, it provides a link between two important regulators of proliferation.

4.1. Uncovering tissue specific roles: restricted ablation of the pocket proteins

The embryonic lethality of the germ line *Rb* knockout precluded a detailed analysis of the contribution of pRB to proliferation, differentiation or tumorigenesis in specific tissues. Different approaches have been used to circumvent this limitation. For years, analysis of mouse chimeras has provided interesting data regarding cell-autonomous and non-cell-autonomous defects in *Rb*-deficient embryos. More recently, the use of the Cre-loxP and Flp-FRT recombination systems allowed the generation

Table 1. Systems of *in vivo* conditional deletion of Rb1

Tissue or cell type	Method for recombinase expression	Conditonal KO strain	Reference
Cardiomyocytes	Tg alphaMHC-Cre	Rb^{F19}	142
Cerebellum progenitors	Tg Engrailed2-Cre	Rb^{F19}	143
Cerebellum external granular cells	Tg GFAP-Cre	Rb^{F19}	88
Cerebellum Purkinje cells	Tg L7-Cre	Rb^{F19}	143
CNS	Tg Nestin-Cre	Rb^{F3}	89
Epidermis	Tg K14-Cre	Rb^{F19}	144
Epidermis	Tg K14-Cre	Rb^{F3}	145
Gastrointestinal tract	Tg villin-Cre	Rb^{F19}	146
Hair cells	Tg Col1A1-Cre	Rb^{F19}	147
Liver	Tg Albumin-Cre	Rb^{F19}	148
Lung epithelium	Tg CC10-rtTA x Tg Tet-Cre	Rb^{F19}	138
Lung	Ad-Cre instillation	Rb^{F19}	149
Melanocyte	Tg TEC-Cre	Rb^{F2}	90
Embrionic tissue	Tg Mox2-Cre	Rb^{F19}	96,79
Myocytes	Tg Myf-Cre, MCK-Cre	Rb^{F19}	100
Ovarian epithelium	intrabursal injection Ad-Cre	Rb^{F19}	150
Pancreas	Tg RIP2-Cre	Rb^{F19}	139
Pituitary IL	Tg POMC-Cre	Rb^{frt19}	91
Prostate	Tg Probasin-Cre	Rb^{F19}	151
Retina	Tg Chx10-Cre	Rb^{F19}	152,153
Retina	Tg IRBP-Cre	Rb^{F19}	154
Retina	Tg Nestin-Cre	Rb^{2LOX}	104
Retina	Tg Pax6-Cre	Rb^{F19}	102,104
Telencephalon	Tg Foxg1-Cre	Rb^{F19}	95

Tg, transgenic mouse; MHC, myosin heavy chain; IL, intermediate lobe; Ad-Cre, adenovirus expressing Cre recombinase; RIP, rat insulin promoter.

of mice carrying conditional alleles of *Rb* that could be inactivated in a cell-type- and time-specific fashion. So far, mouse strains have been generated where loxP sites flank exon 19 (Rb^{F19}) (88), exon 3 (Rb^{2lox}) (89) or exon 2 (Rb^{F2}) (90) of the *Rb1* gene. Additionally, exon 19 has been also engineered with flanking FRT sites (Rb^{frt19}) (91). Because of the growing number of Cre-expressing strains, the Cre-lox lines, and specially the Rb^{F19} generated by Anton Berns´ laboratory (88), have quickly become invaluable reagents for tissue-specific knockout of *Rb* (Table 1).

Data generated by these approaches have challenged previous thoughts about pRB biology. Thus chimeric mice composed by both wild-type and $Rb^{-/-}$ cells soon revealed that *Rb*-null cells were able to contribute to most of adult tissues, including nervous system and the erythroid lineage, two tissues with the most dramatic defects in the germ line $Rb^{-/-}$ embryo (92,93). In particular, ectopic S-phase entry but not cell death was observed in $Rb^{-/-}$ neurons within the CNS, indicating that whereas the proliferation defect was cell autonomous, the apoptosis was not (94). This finding was further confirmed by inactivation of *Rb* with conditional alleles within CNS and PNS (89) or telencephalon (95). Similar findings were observed when *Rb* null embryos were supplied with a wild-type placenta, (96,79) indicating that the prominent apoptosis observed in $Rb^{-/-}$ nervous system was secondary to hypoxia. Moreover, ectopically proliferating neurons were able to differentiate, providing an example where growth arrest is not a prerequisite for terminal differentiation in contrast with other cell types.

In long term survivor $Rb^{-/-}$ embryos with normal placentas erythropoiesis was only mildly affected, with a small fraction of nucleated erythrocytes present, in contrast with the impressive defect in erythroid maturation observed in germ line *Rb* knockout embryos (79). Once again the hematopoietic defects were not the cause of embryo lethality, but secondary to defective placental transport, although an autonomous role for *Rb* in erythroid differentiation has been shown under stress conditions (97).

The stunning finding that most phenotypes in $Rb^{-/-}$ embryos were due to placental defects also revealed a critical role for *Rb* in myogenesis. In fact rescued *Rb* null embryos supplied with wild-type placenta died soon after birth with severe alterations in myoblast differentiation (79). Similar defects were observed in mice carrying a hypomorphic *Rb* minigene, which extends embryo lifespan (98). This *in vivo* observation is also consistent with findings in $Rb^{-/-}$; $Id2^{-/-}$ mice, which survive to term and die with severe reduction in muscle (86). Recently, *in vivo* and *in vitro* conditional inactivation experiments have further defined that *Rb* is required for progression through myogenic differentiation but not for maintenance of a state of terminal differentiation (99,100).

Because human heterozygous for *Rb* develop retinoblastoma, the retina has been subject of detailed study in the different mouse models of *Rb* inactivation. Unlike humans, $Rb^{+/-}$ mice do not develop retinoblastomas but only a mild form of dysplasia. Even mouse chimeras with contribution of *Rb*-null cells in the retina fail to recapitulate the phenotype seen in humans. Instead simultaneous inactivation of a second member of the family is needed. This was first shown in chimeras double deficient for p107 and pRB (101) and later confirmed using Cre-lox systems, with retina-specific deletion of *Rb* into a p107-null background (102,103). Similarly, *Rb* inactivation in the retina also predisposes to retinoblastoma if combined with p130 deficiency (104). The reason of such a different impact in human or mice is unclear, although it could be related with the increase in p107/p130 expression in a compensatory fashion (105,106).

Other tissue-specific knockouts for *Rb* have been generated, uncovering that in many instances *Rb* inactivation causes increased proliferation (skin, liver, cerebellum…) but no tumor development, which requires additional mutations, often disabling the p53 pathway (medulloblastomas, lung carcinomas). Surprisingly enough, other models showed little or none alteration after *Rb* ablation (astrocytes, cardiac muscle, melanocytes), suggesting that functional compensation by the other pocket proteins could be especially important in these particular tissues (see Table 1 for references).

4.2. *Rb* beyond Knockouts

The same recombination-based technologies have allowed the development of several strains of *Rb* knock-in animals. These mutants have also shed some light on pRB functions. As mentioned above, $Rb^{-/-}$ animals showed extensive apoptosis both on the neural system and lenses, suggesting that inhibition of pRB is required for apoptotic cell death. Indeed, a caspase consensus cleavage site is located at the C-terminus of pRB (107). Animals expressing a caspase-resistant pRB (*Rb-MI*) show selective apoptosis resistance, and intestines but not spleens are protected against septic shock (108). MEFs derived from *Rb-MI* animals are protected from TNFRI-induced apoptosis, but sensitive to DNA damage, suggesting that pRB cleavage by caspase is required for TNFRI-induced cell death. The anti-apoptotic function of pRB-MI also raises the possibility that it may promote tumor formation. In fact, crossing *Rb-MI* animals on a $p53^{-/-}$ background resulted in mice with increased colon tumor incidence (109). pRB implications on cell differentiation has been studied by interfering with its ability to modulate the activity of transcription factors. A pRB mutant unable to physically interact with E2F1, E2F2 and E2F3 (R661W) rescues some of the developmental defects associated with the $Rb^{-/-}$ phenotype (110). Specifically, defects on erythrocyte maturation and foetal liver macrophages differentiation are partially rescued on the R661W, whereas no changes on placental transport or rod cell differentiation defects are observed. This indicates that differentiation on some cell types requires a function of pRB that can be separated from its ability to bind E2F1, E2F2 and E2F3. In addition to its binding to the E2F transcription factors, negative regulation of transcription by pRB is also exerted by the interaction with chromatin-modifying enzymes. This interaction takes place through the LXCXE domain of pRB (111). Although mice with mutations in this domain are viable, MEFs derived from these animals show defects on mitosis progression due to a reduction on H4-K20 trimethylation that causes alterations on heterochromatin structure and aneuploidy (112).

4.3. Knockout models for *p130* and *p107*: just needless?

The phenotypes of the knockout of *p107* and *p130* are strikingly dependent on the genetic background in which they are analyzed. While $p107^{-/-}$ mice are apparently normal in pure C57BL/6J or mixed C57/129 backgrounds (81), $p107^{-/-}$ mice on a Balb/cJ background exhibit impaired growth and myeloid hyperplasia, and cells derived from them have a greatly accelerated proliferation rate (113). Likewise, $p130^{-/-}$ mice from the C57BL/6J strain are normal (80), but when the lack of p130 occurs in a Bald/cJ strain, the animals die before birth, showing impaired neurogenesis and myogenesis, with enhanced apoptosis and proliferation in several cell types (114). Significantly, in both cases a single cross of Balb/cJ with C57BL/6 mice made knockout animals viable and fertile, suggesting that one or several modifier genes in the latter strain are sufficient to inhibit the phenotype.

By and large, analysis of the p107 and p130 deficient animals and experiments using cells derived from them, has established firmly these two proteins as negative regulators of the cell cycle at least through inhibition of the E2F family of transcription factors. Moreover, in most strains of mice p107 and p130 have clearly overlapping roles. As it has been stated above, inactivation of one gene gives rise to a viable and fertile mouse in some of the most used laboratory strains, and cells derived from these mice show normal cell-cycle kinetics. In contrast, double *p130/p107* mutant mice die at birth with severe defects in endochondral bone development and deregulated chondrocyte proliferation (80). Later it was shown that this phenotype correlated with the inability of *p107/p130* double mutant chondrocytes to growth arrest and express the chondrogenic transcription factor Cbfa1 (115).

However, phenotypic analysis of these "old" germ-line knockout strains is far from be completed and new and interesting roles for p107 and p130 are still being suggested. Two studies have shown that p107 deficient mice contain elevated numbers of adipogenic (116) and neural precursor cells (117), indicating novel and specific functions for p107 regulating stem cells proliferation, at least in those organs. Similarly, two independent studies have found that p130 cooperates with the cdk inhibitor $p27^{Kip1}$ regulating hematopoietic cell proliferation (57) or mobilization of angiogenic progenitors from the bone marrow (59). Although it is unclear if these two cooperative roles are physiologically related, both suggest a bone fide function for p130 as cdk inhibitor.

Unfortunately, unlike *Rb* no conditional models for *p130* or *p107* exist. Thus, tissue-specific functions for these proteins have been described only in germ-line single- or double-knockout mouse strains, or in combination with tissue-specific *Rb* deletion, as described above.

5. MICE THAT LINK POCKET PROTEINS WITH TUMORIGENESIS

The role of pRB as a tumor suppressor is well-established and, besides familial retinoblastoma, genetic inactivation of pRB has been causally implicated in sporadic cancers such as osteosarcoma, breast, lung or prostate (118).

Although the study of tumor predisposition in germ line *Rb* knockouts is precluded by the phenotype of embryonic lethality, $Rb^{+/-}$ mice in a C57/129 mixed background, develop tumors and succumb within the first year of life. Specifically, $Rb^{+/-}$ mice develop pituitary adenocarcinomas, and thyroid medullary carcinomas with near complete penetrance. As expected for a classical tumor suppressor neoplasias arose after LOH of the wild-type allele (119). Pituitary tumors develop from the intermediate lobe, a tissue found to be tumor-prone in knockout strains for other cell cycle regulatory genes, such as $p27^{Kip1}$ (120,121,122) or $p18^{Ink4c}$ (123). While a possible interpretation is that melanotrophs of the intermediate lobe are exquisitely sensitive to cell cycle deregulation for some unknown reason, recent findings show that wild-type mice of the 129Sv strain show predisposition to melanotroph hyperplasia (124). Thus, the reason of so recurrent tumor

origin in knockout strains could easily be related to a strain-specific predisposition, since most of such mutants are in a 129-pure or enriched background. For this reason, the length of survival and the penetrance of tumors vary dramatically in *Rb* $^{+/-}$ animals between C57BL6 and 129Sv pure genetic backgrounds. Interestingly, when *Rb*$^{+/-}$ animals are crossed into a C57BL6 pure background pituitary tumors are still present, but the origin is the anterior lobe rather than the intermediate lobe. The intermediate lobe of the pituitary is a rudimentary and probably non-functional structure in humans, but tumors of anterior lobe origin are frequent and clinically relevant, so this set of observations place *Rb* inactivation as a possible causal force for human pituitary tumors (125).

5.1. Models for *Rb*-dependent carcinogenesis

The origin and development of intermediate lobe pituitary tumors has been subject of detailed analysis. In *Rb*$^{+/-}$ mice, loss of remaining normal allele of *Rb* occurs early in animal life, during the first 30 days, and those cells undergoing LOH can be detected as "early atypical proliferating" (EAP) cells in the mouse pituitary. However, because melanotrophs are innervated by dopaminergic neurons which negatively regulate their proliferation, abnormally proliferating cells undergo apoptosis (126). In this model, tumors only can develop once those EAP cells acquire additional mutations and become refractory to the inhibitory signals, and this is consistent with the long latency period observed in the development of these tumors. Supporting this model, *Rb*$^{+/-}$*; p53*$^{-/-}$ mice develop tumors with a shorter onset, consistent with the idea that mutation in apoptotic pathways cooperate with *Rb* LOH (127). This attractive model "proliferation plus survival" also provides explanation for how simultaneous inactivation of p53 and *Rb* might cause appearance of medulloblastomas or lung carcinomas in mice. However, p53 mutations are not found in *Rb* null pituitary tumors, so the anti-apoptotic mutations that cooperate with *Rb* and allow EAP cells to further develop into tumors *in vivo* remain unclear. These have to be found among other mutations that collaborate with *Rb*$^{+/-}$ in tumor development. For example, *Rb*$^{+/-}$*; p27*$^{delta51/delta51}$ mice develop melanotroph adenocarcinomas with LOH in the *Rb* locus, and these tumors are more aggressive and their onset occurs earlier (128). Inactivation of p27 function, a common event in human tumors (129), could provide loss of responsiveness to antimitogenic signals and thus an additional advantage to the nascent tumor (130). Another CDK inhibitor, p21, also cooperates with *Rb* restricting tumorigenesis in the pituitary (131), but the mechanism of its contribution has not been explored. Regardless, the collaboration between mutations in *Rb* and CDK inhibitors reveals that CDK activity has targets different than pRB important for tumor development, and the related molecules p130 and p107 are obvious suspects.

Loss of *p19*^{Arf} also accelerates appearance of intermediate-lobe tumors in *Rb*$^{+/-}$ mice, adding Arf to the list of genes shown to cooperate with *Rb* inhibiting pituitary carcinogenesis by genetic approaches. In this case, Arf seems to act in a p53-independent fashion, since loss of Arf does not affect cell death rates but increases the appearance of EAP cells (132).

Similar models for retinoblastoma origin have been postulated. As for pituitary tumors, one "death model" suggests that mouse retinal progenitors that lose *Rb* and *p107* (or *Rb* and *p130*) acquire infinite proliferative capacity but, because they are death-prone, must accumulate an antiapoptotic mutation in order to originate a tumor. This model was originally supported by observations of cell death in several *Rb*-deficient cell types in the retina, but it is being replaced for a novel view where *Rb*/*p107* loss does not affect progenitor proliferation, but perturbs cell cycle exit associated to terminal differentiation. According to this new "differentiation model" retinoblastoma arises from specific precursors with extended proliferative capacity, but not death-prone. Thus, additional lesions driving retinoblastoma would overcome growth arrest, not apoptosis (133).

In the same way that genetics have uncovered mutations that collaborate with *Rb* inactivation accelerating tumorigenesis, mouse models have provided important information about genes lying downstream of *Rb* regarding tumor suppression. The obvious targets of *Rb* action are the E2F transcription factors. According to the script, *Rb*$^{+/-}$*; E2F1*$^{-/-}$ mice show prolonged survival with reduced incidence and longer latency of pituitary and thyroid tumors (134). Not so obvious were the phenotypes found for other members of the E2F family suggesting that the functional relationships between pRB and E2Fs could be more complex than we envision. Thus, crossing *Rb*$^{+/-}$ mice into a E2F3 null background caused reduction on pituitary tumors but enhanced tumorigenesis on thyroid as well as pancreas, endometrium, or adrenal gland (85). In contrast, loss of a "repressor E2F" such as E2F4 over *Rb* heterozygosis leads to a reduction in both pituitary and thyroid tumors, a phenomenon that has been linked to the compensatory ability of freed p130/p107 to bind and inhibit the "activator" E2F1 (21).

6. *p130* AND *p107* IN CANCER: ONLY A BACKUP FOR *Rb*?

Unlike *Rb*, genetic inactivation by mutation in *p107* or *p130* loci is not a common event in human cancers. p130 lies on the human chromosome 16q12.2, a region lost in a fraction of breast, ovarian or prostatic carcinomas. More importantly, low p130 protein levels are associated with bad prognosis in endometrial and lung cancer, as well as melanomas (135), suggesting that, like p27, p130 can behave as a tumor suppressor protein, rather than a tumor suppressor gene. So far, there is no evidence of a similar suppressive role for p107 from cytogenetics or expression studies.

Single knockouts for *p130* or *p107* show no predisposition to spontaneous tumor formation. Similarly, no development of neoplasia has been reported even in *p107*$^{+/-}$*; p130*$^{-/-}$ or *p107*$^{-/-}$*; p130*$^{+/-}$ animals (80,81) in a mixed genetic background. But even when the knockout phenotypes do not provide support a role of these pocket

Table 2. Phenotypes of the different strains of mutant mice for the genes of Rb family

Mouse strain	Phenotype	References
Rb$^{-/-}$	Emrbyonic lethal. Abnormal placenta development that causes hypoxia, altered erythropoiesis and apoptosis on CNS. Increased lens proliferation and apoptosis.	76,77
Rb$^{-/-}$ *Chimeras*	Chimeras are viable. Ectopic S-phase entry but not cell death in *Rb*$^{-/-}$ neurons within the CNS. Cataracts, hyperplasia of the adrenal medulla. Pituitary and thyroid tumors.	94,92,93
Rb$^{-/-}$*; p107*$^{-/-}$	Earlier embryonic lethality. Accelerated apoptosis in the liver and CNS relative to Rb$^{-/-}$ embryos.	81
Rb$^{-/-}$*; p107*$^{-/-}$ *Chimeras*	Chimeras viable. Retinoblastoma, pituitary and thyroid tumors.	101
Rb$^{-/-}$*; p130*$^{-/-}$*Chimeras*	Early death. Retinoblastoma and predisposition to pheochromocytoma and lung hyperplasia.	141
Rb$^{-/-}$*; E2F1*$^{-/-}$	Embryonic lethal. Partial rescue of the Rb$^{-/-}$ phenotype: significant suppression of apoptosis and S phase entry in CNS, PNS and lens. Defects on skeletal muscle and lung development.	83,84
Rb$^{-/-}$*; E2F2*$^{-/-}$	Partial rescue of the unscheduled proliferation observed on Rb$^{-/-}$ lens, retina and CNS. No effect on apoptosis in lens, retina or CNS.	83
Rb$^{-/-}$*; E2F3*$^{-/-}$	Embryonic lethal, but increase in lifespan compared with Rb$^{-/-}$ embryos. Partial or complete rescue of unscheduled proliferation on lens and retina, and complete rescue on CNS. Suppression of apoptosis in lens and CNS. Partial rescue of hematopoietic defects. Altered muscle, lung and cardiac development.	83,155
Rb$^{-/-}$*; Id2*$^{-/-}$	Neonatal lethality. Partial to total rescue of neurological and haematopoietic defects observed on Rb$^{-/-}$ animals. Severe reduction of muscle tissue.	86
p107$^{-/-}$ *C57BL/6J or mixed C57/129*	Viable and fertile. Increased number of neural progenitors.	81
p107$^{-/-}$ *Balb/cJ*	Viable but impaired growth. Ectopic myeloid hyperplasia in the spleen and liver.	113
p130$^{-/-}$ *C57BL/6J*	Normal development with no obvious phenotype.	80
p130$^{-/-}$ *Balb/cJ*	Embryonic lethal E11-13. Impaired neurogensis and myogenesis. Enhanced proliferation and apoptosis in several cell types.	114
p107 $^{-/-}$*; p130*$^{-/-}$	Neonatal lethality. Severe defects in endochondral bone development and deregulated chondrocyte proliferation. Shortened limbs.	80,115
Rb$^{+/-}$	Viable. Pituitary and thyroid medullary carcinomas with near complete penetrance. Tumors show loss of heterozygosity of the wild type allele.	119,77,126
Rb$^{+/-}$*; p107*$^{-/-}$	Poor viability and growth retardation. Multiple displastic lesions of the retina. Tumor spectrum similar to Rb$^{+/-}$ mice.	81
Rb$^{+/-}$*; p107*$^{-/-}$ *Chimeras*	Chimeras have increased viability compared to pure *Rb*$^{+/-}$*; p107*$^{-/-}$. Wider tumor spectrum than Rb$^{+/-}$ mice. Occasional retinal dysplasia.	141
Rb$^{+/-}$*; E2F1*$^{-/-}$	Extended lifespan with reduced incidence and longer latency of pituitary and thyroid tumors.	134
Rb$^{+/-}$*; E2F3*$^{-/-}$	Viable with significantly extended lifespan compared to Rb$^{+/-}$. Altered tumor spectrum with suppression of pituitary tumors development and increase on medullary thyroid carcinomas incidence and metastasis.	85
Rb$^{+/-}$*; E2F4*$^{-/-}$	Extended lifespan compared to Rb$^{+/-}$ with a nearly complete suppression of both pituitary and thyroid tumor incidence.	21
Rb$^{+/-}$*; p27*$^{delta51/delta51}$	Viable with reduction in lifespan related to Rb$^{+/-}$. Intermediate lobe and medullary thyroid carcinomas with complete penetrance and loss of heterozygosity. Tumors more aggressive and with shorter latency periods.	128
Rb$^{+/-}$*; p21*$^{-/-}$	Viable. Early mortality compared to Rb$^{+/-}$. Acceleration of intermediate lobe tumors.	131
Rb$^{+/-}$*; Arf*$^{-/-}$	Reduction on lifespan with strong acceleration of intermediate lobe pituitary tumorigenesis compared to Rb $^{+/-}$ mice.	132
Rb$^{+/-}$*; p53*$^{-/-}$	Reduced lifespan and earlier onset of pituitary tumors, with LOH on Rb but not on p53. Increased tumor incidence, with development of pinealoblastomas, islet cell tumours, bronchial epithelial hyperplasia and retinal dysplasia	127
Rb-MI	Viable. Resistance to apoptosis in selective tissues in response to specific death stimuli.	108
Rb$^{R654W/ R654W}$	Embryonic lethal, but increase in lifespan compared with Rb$^{-/-}$ mice associated with improved erythrocyte and fetal liver macrophage differentiation. No rescue of differentiation defects affecting retinae.	110
Rb1$^{deltaL/deltaL}$	Viable, fertile and obtained at nearly the expected Mendelian ratio.	112

proteins restraining tumor origin and/or progression, several data argue otherwise. Most of the data has been gathered from cell culture systems: for instances, the three pocket proteins conspire collaboratively to maintain the G1 checkpoint as evidenced in the triple knockout fibroblasts (48,49). Furthermore, the transformation of mouse fibroblasts mediated by the viral oncoprotein SV40 large T-antigen requires inactivation of the three family members (136,137). Similar genetic evidences were obtained *in vivo* because expression of LTAg causes more severe alterations than *Rb* inactivation alone in several tissues such as lung (138) or prostate epithelium (139).

The first evidence of a tumor suppressor role for p107 *in vivo* came, as mentioned above, from *Rb*/*p107* double deficient chimeras, which, in contrast to single *Rb* null, do develop retinoblastoma (101). A similar role for p130 has been recently discovered, since *Rb* inactivation in *p130* deficient retinas also predisposes to retinoblastoma (102,104).

Because the at least partially redundant role between p107/p130 and pRB regulating tumor appearance, it has been proposed that p130 and p107 could function as tumor suppressors in combination with other mutations (140), most likely *Rb* deficiency or synonymous alterations. This hypothesis is consistent with the existence of modifier genes that affect the phenotypic manifestation of *p130* or *p107* deletion in a background-specific fashion (114,113) and that could similarly affect the suppressive properties of these pocket proteins.

Indeed, so far the evidences for antioncogenic functions of p130 and/or p107 have been always linked to situations where pRB function is disabled. The collaborative role of p130/p107 with pRB preventing retinoblastoma is just an example. Crossing the germ line knockouts or analyzing double deficient chimeras has also revealed cooperation between *Rb* and each of its relatives *p107* and *p130* (141). Interestingly, *p130* loss cooperated with *Rb* originating retinoblastomas, pheochromocytomas

and small-cell lung hyperplasia, while *p107* deficiency caused a significantly expanded tumor spectrum, affecting gastrointestinal system, thymus, thyroid, etc.

Analysis of $Rb^{+/-}$; $E2F4^{-/-}$ mice revealed that p130 and p107 are able to suppress tumorigenesis caused by loss of *Rb* through their ability to buffer the deregulated free E2F1 activity (21). Moreover, data generated in our laboratory indicates that inactivation of other pathways, such as p27 or p53, can also cooperate with the tumor suppressor functions of p130 and p107, even in presence of a functional pRB (our unpublished observations). Furthermore, *p130* null mice show increased tumor development after challenge with chemical carcinogens (unpublished).

How p130 and p107 perform their antioncogenic functions remains to be solved. There is genetic proof that part of the suppressive role of pRB is dependent on the capability to inhibit E2F activity. Based on the shared ability of the pocket proteins to bind and inhibit most of E2F species, a likely scenario is that p130 and p107 work through this mechanism. However, we lack of genetic evidences to sustain this hypothesis. Alternatively, p130 or p107 could contribute to maintain tissues in a non-malignant state through their CDK-inhibitory function or by regulating transcription in an E2F-independent fashion, perhaps through chromatin remodelling. New and exciting research projects are granted to clarify this important issue.

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