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Review

Retinoblastoma family proteins: insights gained through genetic manipulation of mice

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Abstract. The retinoblastoma (Rb) gene was identified as the first tumor suppressor gene two decades ago. Since this initial discovery, it has become clear that deregulated Rb function constitutes a hallmark of human malignancies. Rb is a well-established regulator of the cell cycle. Rb has also been implicated in playing a role in a wide variety of cellular processes including DNA repair, cellular senescence, cell fate determination and apoptosis. Animals lacking Rb and/or its family members p107 and p130 have led scientists to uncover new and exciting roles for this protein family in development as well as tumor suppression. The ability to ablate Rb in a temporal and cell-type-specific manner has offered further, often unexpected, insights into Rb function. This review summarizes the phenotypic consequences of Rb family ablation in mice, and discusses how these findings contribute to the increasingly complex picture of Rb family function in development and tumor suppression.

Key words. Rb; p107; p130; Cre-LoxP system; mouse models.

Introduction

The pediatric eye tumor retinoblastoma was noted more than 50 years ago to occur sporadically in some patients (55–65%), but to be inherited in others (35–45%) [1, 2] This initial observation into the genetics of retinoblastoma offered a unique opportunity to uncover the molecular mechanisms underlying a form of human cancer. In 1986, the retinoblastoma gene (Rb) was cloned and subsequently validated as the first tumor suppressor gene [2–4]. Since its initial discovery, Rb has been found to be inactivated in many additional cancers [5–7]. Indeed, it is now evident that deregulation of Rb constitutes one of the hallmarks of human malignancies and is a focal point of cancer research [8–11]. Thus, elucidating the gene underlying a rare pediatric eye tumor has defined a new class of cancer-associated genes and has greatly contributed to our understanding of human malignancies.

Rb is a well-established regulator of the cell cycle [5, 7, 12, 13]. The Rb protein is expressed in many, if not all, tissues and is regulated in a cell-cycle-dependent manner, consistent with it having a general role in cell cycle regulation. Current models propose that Rb controls cell cycle progression through interactions with the E2F family of transcription factors [14–18]. Accordingly, E2F-binding sites are found in the promoters of many genes known to be important for cell cycle progression. Rb is capable of repressing these genes by at least two different mechanisms. First, Rb can bind to E2F transcription factors and block their ability to activate gene transcription. Second, Rb-E2F complexes can act as active repressors by recruiting chromatin-modifying complexes including histone deacetylases and methylases, DNA methyltransferases and ATP-dependent chromatin-remodeling enzymes. Accumulating evidence indicates that Rb-mediated repression of E2F-regulated genes is important in cell cycle regulation. However, it is still unclear which *in vivo* biologi-

cal functions of Rb are achieved through its effects on E2F-mediated gene transcription.

Rb has been implicated in regulating a wide variety of cellular processes in addition to its role in the cell cycle [5, 12, 19–21]. These processes include DNA replication, mitosis, DNA repair, DNA damage checkpoint control, cellular senescence, differentiation and apoptosis. Additionally, Rb-E2F complexes may also be important for pattern formation during development. Consistent with this idea, Rb-E2F is required for establishing embryonic asymmetry and organ morphogenesis in *Caenorhabditis elegans* [22, 23], oocyte dorsoventral polarity in *Drosophila* [24] and ventral and posterior cell fate determination during *Xenopus* embryogenesis [25]. The mechanisms underlying these functions are currently unclear but have been shown to be independent of abnormalities in cell cycle control in some cases. Interestingly, Rb-E2F complexes act as transcriptional repressors to antagonize Ras signaling during vulval development in *C. elegans* leading to changes in cell fate [26]. Additionally, E2F acts as an important regulator of region-specific Hox gene expression during *Xenopus* development [25]. These studies suggest that Rb-E2F complexes play a more direct role in regulating cell fate rather than simply permitting differentiation by establishing cell cycle arrest.

Much of our current understanding of Rb function has resulted from molecular studies done in cells in culture. However, analysis of animals specifically lacking Rb and its family members p107 and p130 has provided key insights into the roles pocket proteins play in development and tumor suppression (tables 1, 2). Temporal and cell-type-specific Rb ablation has recently contributed even further insights into Rb function *in vivo* (table 3). The goal of this review is to provide a concise overview of the phenotypic consequences of Rb family ablation in mice, and to discuss general concepts that have emerged from genetic manipulation of Rb family proteins *in vivo*.

The Rb regulatory pathway plays an essential role in tumor suppression

Rb limits cell proliferation by arresting cells in the G1 to S phase of the cell cycle (fig. 1). This growth inhibitory function is regulated by phosphorylation [7, 16]. Rb is inactivated in proliferating cells through phosphorylation by the G1 cyclin-dependent kinase cyclin-D-cdk4/6 in collaboration with cyclin E-cdk2. The activity of cyclin-D-cdk 4/6 is in turn regulated by the cyclin dependent kinase inhibitor p16. Accordingly, Rb is maintained in an inactive hyperphosphorylated state in cells with increased cyclin-D-cdk4/6 activity or loss of p16 function.

Rb, cyclin-D-dependent kinases and p16 constitute a pathway that is disrupted in virtually all human cancers [5–7]. Indeed, the nearly universal detection of mutations in particular components of this pathway has lead to the speculation that disabling the Rb pathway may be required for the generation of malignant cells. Rb mutations and p16 loss are mutually exclusive events in carcinomas, confirming that these proteins function in a common regulatory pathway. Interestingly, particular components of the Rb pathway are preferentially inactivated in distinct malignancies (fig. 1). Rb gene mutations are frequently observed in only a small subset of human cancers, namely retinoblastoma and small-cell lung cancers, whereas loss of p16 function is more common and occurs in a wider variety of tumors. Cyclin D/cdk4 overexpression is most frequently detected in breast cancer, mantle cell lymphomas and glioblastomas. Why different components of the Rb pathway are preferentially targeted in distinct tumor types remains unclear.

Rb belongs to the pocket protein family

Rb belongs to a family of proteins that also includes p130 and p107 [27–29]. This protein family is defined by a highly conserved pocket domain that is critical for biologic function (fig. 2). All three proteins interact with viral oncoproteins, inhibit E2F-responsive promoters, actively repress gene transcription, are regulated by cdk-dependent phosphorylation and arrest cells in the G1 phase of the cell cycle. The pocket proteins also show functional overlap during development (table 1). The structural similarities shared by this family likely explain why the pocket proteins have overlapping functions.

Features that distinguish p107 and p130 from Rb include differences in expression pattern, interactions with the various E2F family members and associations with cyclin/cdk complexes [12, 15, 17, 28] (fig. 2). p130 is highly expressed in quiescent and differentiated cells and its levels decline as cells are stimulated to proliferate. In contrast, p107 is preferentially expressed in proliferating cells and is generally low in quiescent and differentiated cells. Rb is expressed in most, if not all, cell types and can be detected in proliferating, quiescent and differentiated cells. The physiological significance of these dynamic fluctuations in pocket protein expression is currently unknown.

Another distinction between p107/p130 and Rb is in their preferential binding to specific E2F family members (fig. 2). Eight E2F family members have been identified to date [5, 14, 18, 30, 31]. The family members can be divided into two general subgroups: ‘activating’ and ‘repressing’ E2Fs. In general, the activating E2Fs are periodically expressed during the cell cycle and promote cell cycle progression, whereas the repressing E2Fs are expressed throughout the cell cycle and are believed to be required for cell cycle exit and differentiation. p107 and p130 associate with the repressing E2Fs, namely E2F4

Table 1. Phenotypes in pocket protein deficient mice

Genotype	Lethality	Major phenotypes[1]	References
$Rb^{-/-}$	E13.5–E15.5	Increased proliferation and apoptosis in CNS and PNS; defective hematopoiesis; increased lens proliferation and apoptosis and loss of late differentiation markers.	67, 82–84
$p107^{-/-}$		Increased CNS neural progenitor cell proliferation and apoptosis; subtle thickening of long bones; impaired growth and myeloproliferative disorder in Balb/cJ background.	85–87
$p130^{-/-}$	E11–13 or normal	Normal or lethality with aberrant neural, muscle and heart development in Balb/cJ background.	85, 88
$p107^{-/-};p130^{-/-}$	birth	Aberrant endochondral bone development; increased epidermal proliferation and aberrant differentiation; decreased hair follicles and developmental delay in hair, whisker and tooth formation.	85, 89
$Rb^{-/-};p107^{-/-}$	E11–13	**Retinoblastoma**; enhanced apoptosis in liver and CNS. Chimeric mice obtained at low frequency and develop retinoblastoma.	60, 90, 91
$Rb^{-/-};p130^{-/-}$	E11–13	Enhanced apoptosis in CNS and liver. Chimeric mice develop lung neuroendocrine hyperplasia, **retinoblastoma and pheochromocytoma.**	19, 60
$Rb^{-/-};p53^{-/-}$	E13.5–E15.5	Rescue of apoptosis in CNS and lens; enhanced apoptosis in PNS; enhanced proliferation in CNS and PNS; no effect on proliferation and differentiation in lens.	84, 92
$Rb^{-/-};E2F1^{-/-}$	E17.0	Partial/complete rescue of proliferation and apoptosis in CNS, PNS and lens; partial rescue of hematopoiesis; aberrant muscle and lung development.	93, 94
$Rb^{-/-};E2F2^{-/-}$	not given	Partial rescue of proliferation in CNS, retina and lens; no effect on apoptosis in CNS, retina and lens.	93
$Rb^{-/-};E2F3^{-/-}$	E18	Partial/complete rescue of proliferation in CNS, PNS, retina and lens and of apoptosis in CNS and PNS; rescue or increased apoptosis in lens; no effect on apoptosis in retina; partial rescue of hematopoiesis; aberrant muscle, lung and cardiac development.	93, 95
$Rb^{-/-};ARF^{-/-}$	not given	Enhanced PNS apoptosis and proliferation; mild rescue of lens apoptosis; no effect on CNS proliferation or apoptosis.	72
$Rb^{-/-};Id2^{-/-}$	birth	Rescue of erythropoiesis; rescue of neurogenesis except slightly increased apoptosis in PNS; aberrant muscle differentiation, increased proliferation and apoptosis.	65
$Rb^{-/-};N\text{-}ras^{-/-}$	E16.5–18.5	Enhanced muscle differentiation with no effect on ectopic proliferation and apoptosis; no effect on CNS, PNS and lens phenotypes.	96
$Rb^{-/-};K\text{-}ras^{+/-}$	E14.5–16.5	Enhanced muscle differentiation with no effect on ectopic proliferation and apoptosis; no effect on CNS and PNS phenotypes.	41
$Rb^{-/-};Casp3^{-/-}$	E13.5–E15.5	Rescue of PNS apoptosis and neuronal differentiation; no effect on apoptosis in CNS and hematopoietic cells.	97
$Rb^{-/-};Apaf1^{-/-}$	E13.5 or earlier	Rescue of apoptosis in CNS and lens; partial rescue of apoptosis in PNS and muscle.	98

CNS, central nervous system; PNS, peripheral nervous system.
[1] Tumor phenotypes are in bold type; phenotypes in double-mutant mice are given in reference to the phenotype in $Rb^{-/-}$ mice.

and E2F5. In contrast, Rb can interact with both subgroups but preferentially associates with the activating E2Fs, E2F1–3. The remaining E2F family members do not bind pocket proteins and regulate transcription in a pocket-protein-independent manner.

Analysis of cells lacking pocket protein family members indicates that distinct E2F target genes are regulated by p107/p130 and Rb. Many known E2F-responsive genes are deregulated in p107/p130 double-null murine embryo fibroblasts (MEFs) [32–34]. In contrast, Rb deficiency results in deregulation of a much smaller set of E2F target genes, namely cyclin E and p107. A more global examination of pocket-protein-specific gene targets has been done using DNA microarray analysis [35]. These studies provide further evidence that the absence of p107/p130 or Rb results in distinct changes in gene expression. Interestingly, the genes characterizing the Rb null state include a substantial number of genes encoding DNA replication and cell cycle regulatory proteins. In contrast, the genes identified as deregulated in the cells lacking

Table 2. Phenotypes in Rb Heterozygous Mice

Genotype	Tumors	Other phenotypes[1]	References
$Rb^{+/-}$	pituitary thyroid pheochromoctyoma	islet cell hyperplasia lung neuroendocrine hyperplasia	60, 66–68, 99
$Rb^{+/-}$;$p107^{-/-}$	pituitary chimeric mice cecal adenocarcinoma sarcomas lymphosarcoma thymoma ovary, testis thyroid, adrenal lung	retinal dysplasia ~75% lethality by 3 weeks of age severe growth retardation and vaginal atresia chimeric mice live and get additional tumors	60, 90, 91
$Rb^{+/-}$;$p53^{-/-}$	pituitary thyroid islet cell pinealoblastomas	reduced survival novel tumors seen in double mutants lung neuroendocrine hyperplasia retinal dysplasia	68, 100
$Rb^{+/-}$;$E2F1^{-/-}$	pituitary thyroid	prolonged survival reduced pituitary and thyroid tumorigenesis adrenal medullary hyperplasia	101
$Rb^{+/-}$;$E2F3^{-/-}$	pituitary thyroid islet cell pheochromoctyoma parathyroid pinealoblastoma uterine endometrium	prolonged survival reduced pituitary tumorigenesis enhanced thyroid tumorigenesis and metastasis	102
$Rb^{+/-}$;$E2F4^{-/-}$	pituitary thyroid	prolonged survival decreased tumorigenesis in pituitary and thyroid	103
$Rb^{+/-}$;$ARF^{-/-}$	pituitary thyroid	reduced survival accelerated pituitary tumor development and proliferation	104
$Rb^{+/-}$;$Id2^{-/-}$	pituitary	prolonged survival decreased proliferation and angiogenesis, and increased differentiation of tumors	105
$Rb^{+/-}$;K-$ras^{+/-}$	pituitary thyroid	prolonged survival increased differentiation and decreased proliferation of pituitary tumors	41

[1] Phenotypes in double-mutant mice are given in reference to the phenotype in $Rb^{-/-}$ mice.

p107/p130 are largely devoid of DNA replication and cell cycle genes, but rather encode proteins involved in regulation of cell growth, maintenance of the extracellular matrix and signaling activities linked to the extracellular matrix.

Although these studies suggest that Rb may have a dominant role in cell cycle regulation, other studies challenge this notion. First, Rb itself is not essential for cell cycle control [36]. Furthermore, Rb is rarely if ever detected at the promoters of E2F target genes *in vivo* [5, 14, 15], and p107/p130 rather than Rb recruit histone deacetylases to E2F-responsive promoters in normal fibroblasts [37]. Finally, the gene expression profile that characterizes Rb-deficient MEFs readily distinguished tumors that develop in Rb-deficient mice from normal tissues and from tumors derived from mutations in alternate pathways [35]. This finding suggests that Rb has similar transcriptional effects in diverse cell types. However, Rb null mice and humans with germline Rb mutations develop tumors in very limited cell types. Thus, Rb may play an important role in cell cycle regulation only under certain conditions. Alternatively, Rb may have essential tumor-suppressive functions unrelated to its role in cell cycle control. Taken together, these studies highlight the current gap between molecular studies of Rb-family-mediated gene regulation and pocket-protein-dependent phenotypes *in vivo*.

An additional biochemical property that clearly differentiates p107/p130 from Rb is their ability to form complexes with cyclin A/cdk2 and cyclin E/cdk2. Rb lacks the binding domain required for this interaction. The bio-

Table 3. Phenotypes in conditional Rb ablated mice.

Tissue	Promoter	Major phenotypes[1]	References
Embryo (wild-type placenta)	Mox2 (same results with tetraploid aggregation)	Lethal at birth; increased proliferation and no apoptosis in CNS; increased proliferation and apoptosis at E13.5 but ameliorated by E15.5 in PNS; increased proliferation and apoptosis and disorganization in lens; normal retina and liver hematopoiesis; mild delay in erythrocyte terminal differentiation; skeletal muscle with hypoplastic and dysplastic myofibers.	42, 43
Retina	Nestin paternally derived	Lethality at birth; increased proliferation and apoptosis $Rb^{-/-}$;$p53^{-/-}$ Retinal dysplasia.	106
	Nestin maternally derived (mosasic pattern)	Viable at ~41% of expected frequency; apoptosis and disorganization. $Rb^{-/-}$;$p107^{-/-}$ Lethality prior to weaning; retinal dysplasia and apoptosis. **$Rb^{-/-}$;$p130^{-/-}$ Retinoblastoma**; reduced viability; increased apoptosis and mitosis.	106
	IRBP (photoreceptor cells and pinealocytes)	**Pituitary tumors.** **$Rb^{-/-}$;$p53^{-/-}$ Pineal tumors.** $Rb^{-/-}$;$p107^{-/-}$ and $Rb^{-/-}$;$p53^{-/-}$;$p107^{-/-}$ No additional defects.	107
	Pax 6 (distal retina progenitor cells)	Increased proliferation and apoptosis with selective loss of photoreceptor, ganglion and bipolar cells but not amacrine, horizontal and Muller cells; retinal disorganization. **$Rb^{-/-}$;$p107^{-/-}$ Retinoblastoma.**	54, 106
Pituitary	POMC	**Pituitary tumors.**	69
Nervous system and lens	Nestin	Lethality at birth; increased brain size with increased proliferation but not apoptosis; increased proliferation and apoptosis in PNS and lens; normal erythropoiesis; disorganized myotubes with increased proliferation.	44
Telencephalon	Foxg1	Lethality at birth; enlarged telencephalon; mildly increased apoptosis at E13.5 resolved by E16.5; increased proliferation; expression of early differentiation markers.	108
Astrocytes and cerebellum	GFAP	Normal. **$Rb^{-/-}$;$p53^{-/-}$ Medulloblastoma.**	56
Cerebellum	Engrailed2 (progenitor cells)	Increased proliferation and p53-independent apoptosis; aberrant differentiation. $Rb^{-/-}$;$p107^{-/-}$ Phenotype exacerbated.	109
	L7 (Purkinje cells)	Increased proliferation without apoptosis; normal differentiation and migration; dysplasia.	109
Prostate epithelium	Probasin	Focally increased epithelial proliferation and hyperplasia.	110
Lung epithelium	CC10	Increased proliferation and apoptosis at birth that resolves; neuroendocrine hyperplasia.	75
	adult (IT Cre)	Normal. **$Rb^{-/-}$;$p53^{-/-}$ Small cell lung carcinoma.**	76
Hepatocytes	Albumin and adult (IV Cre)	Increased proliferation that resolves; nuclear pleomorphism and increased ploidy.	111
Epidermis	Keratin 14	Increased basal and suprabasal proliferation; increased centrosome numbers; failure to arrest in response to ionizing radiation; altered differentiation. $Rb^{-/-}$;$p107^{-/-}$ Lethal at postnatal day 8–11; growth retarded; hairless; apoptosis in follicular epithelium; enhanced suprabasal proliferation and aberrant differentiation.	40, 112
Skeletal muscle	Myf5	Lethality at birth; increased proliferation and apoptosis; reduced muscle mass, disorganization and lack of terminal differentiation.	113, 114
	MCK	Normal development and regeneration after injury.	113
Cardiac muscle	αMHC	Normal. $Rb^{-/-}$;$p130^{-/-}$ lethality (52%) by 6 months; increased heart size, myocyte proliferation and expression of fetal proteins; no apoptosis.	55
Inner-ear hair cells	Collagen 1A1	Perinatal lethality; increased proliferation; normal or increased apoptosis; intact differentiation and function.	39, 115

[1] Tumor phenotypes are in bold type.
IT, intratracheal;
IV, intravenous.

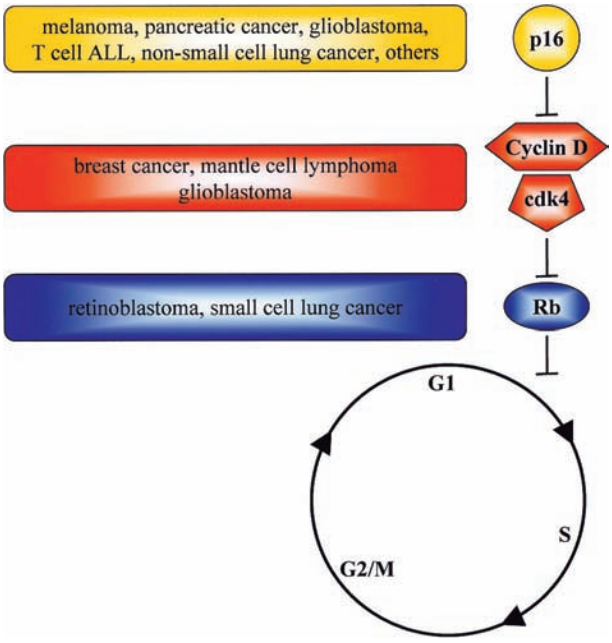

Figure 1. Rb pathway in human cancer. Rb blocks cells in the G1 to S phase transition of the cell cycle. Upon mitogenic stimulation, Rb is inactivated by cyclin-D/cdk4-dependent phosphorylation and cells progress into S phase. Cyclin D/cdk4 activity is, in turn, negatively regulated by the cyclin-dependent kinase inhibitor p16. Mutations in the Rb pathway are frequently detected in human cancers. The different malignancies where loss of p16 or Rb, or overexpression of cyclin-D or cdk4 occurs with high frequency are given next to each component of the pathway.

logical consequences of this interaction are not clear. It has been suggested that p107 and p130 may act as cdk inhibitors by sequestering the proteins or reducing their kinase activity. Alternatively, the interaction may more efficiently target kinase activity to their substrates, including the pocket proteins themselves.

E2Fs, cyclin A/cdk2 and cyclin E/cdk2 are the best studied binding partners for p107 and p130. However, several additional proteins have been reported to interact with p107 and p130 [27, 28]. These proteins include transcription factors important in proliferation as well as differentiation and homeotic processes. In almost all cases, proteins that bind to p107 and/or p130 have also been reported to associate with Rb. Over 100 proteins have been reported to associate with Rb and this list continues to grow [27, 38]. Not all of these proteins have been assayed for their ability to interact with p107 and p130. It is currently unclear which of these interactions occur under physiological conditions, and more importantly, which of these potential partners contribute to pocket protein function *in vivo*.

Regulatory roles of Rb in proliferation, apoptosis and differentiation can be functionally separated

The pocket protein family has well-established roles in regulating cellular proliferation, apoptosis and differentiation. It was initially hypothesized that the effects on survival and differentiation were secondary to the changes in cell cycle control that resulted from loss of Rb family function. However, this does not appear to be the case. There are now several examples where alterations in proliferation can be functionally separated from apoptotic and differentiation phenotypes (tables 1, 3). For example, Rb-deficient ear inner hair cells retain function and a differentiated phenotype despite increased proliferation [39]. Differentiation and proliferation are also uncoupled processes in Rb-deficient skin as evidenced by concomitant expression of differentiation markers and aberrant DNA synthesis [40]. Furthermore, Rb/p107 null keratinocytes show inappropriate prolifer-

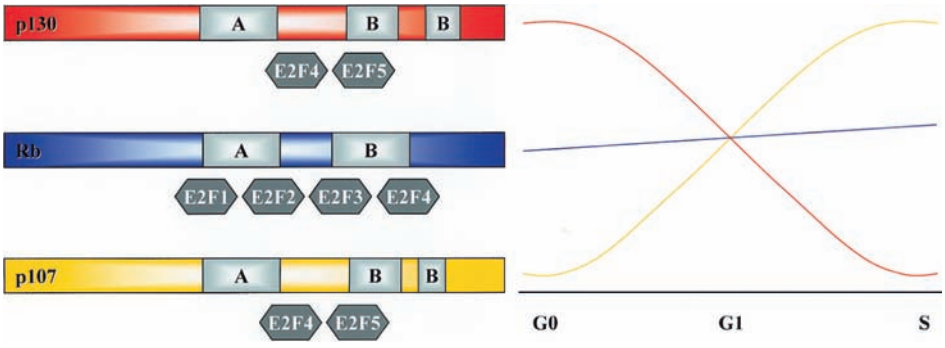

Figure 2. Pocket protein family. p130, Rb and p107 have extensive homology in the 'pocket' which is a bipartite domain comprised of regions A and B separated by a spacer region. The cyclin-A/E-binding site unique to p107 and p130 maps to the spacer region. The pocket is also required for interactions with many other proteins including the E2F transcription factors. Rb preferentially interacts with the activating E2Fs (E2F1, E2F2 and E2F3), whereas p107 and p130 interact with the repressing E2Fs (E2F4 and E2F5). Rb also interacts with E2F4. The pocket proteins are differentially expressed throughout the cell cycle. Rb expression is relatively steady thoughout the cell cycle, whereas p130 expression is highest in arrested cells and p107 expression peaks during S phase.

ation but maintain expression of markers of terminal differentiation. Rb null embryos show enhanced muscle differentiation in a K-ras heterozygous background as compared to a wild-type K-ras background [41]. Enhanced muscle differentiation in these animals occurs in the absence of changes in cell cycle regulation. Thus, proliferation and differentiation phenotypes are uncoupled in a variety of cell types suggesting that these two cellular processes are, at least in part, independently regulated by Rb.

As with differentiation, the aberrant apoptosis resulting from Rb deficiency does not directly correlate with aberrant cell cycle control (tables 1 and 3). In the central nervous system, the aberrant apoptosis in Rb null embryos can be rescued by providing the embryo with a wild-type placenta [42, 43]. In contrast, marked abnormalities in cell cycle control remain. These studies suggest that the apoptosis results from inadequate placental function whereas the aberrant cellular proliferation represents a cell-autonomous phenotype. Consistent with this notion, conditional Rb ablation in the nervous system resulted in brain enlargement due to increased proliferation in the absence of apoptosis [44].

Mice nullizygous for Rb and E2F1, 2 or 3 show preferential rescue of proliferation and/or apoptosis providing further support that these two processes are independently regulated (table 1). E2F1 deficiency leads to suppression of apoptosis but has less of an effect on restoration of normal cell cycle control. In contrast, E2F2 deficiency results in partial restoration of cell cycle control but has no influence on apoptosis. Significantly, mutation of a single E2F3 allele almost completely suppressed apoptosis in the central nervous system and lens. In contrast, two distinct classes of embryo were noted with regard to proliferation: (i) embryos with near complete suppression of inappropriate proliferation and (ii) embryos with no suppression of ectopic proliferation. Differential suppression of proliferation in these two classes was attributed to strain-specific modifiers in the mixed genetic background. Taken together, these analyses show that apoptosis is not merely a default response triggered by inappropriate proliferation, and that the aberrant apoptosis, differentiation and proliferation arising from loss of Rb are independently regulated.

Pocket proteins have cell-lineage-specific functions

The current model of Rb function suggests a critical role in cell cycle regulation operative in all cell types. Consistent with this notion, pocket proteins are fairly ubiquitously expressed throughout development and in adult tissues [45–49]. It was therefore initially surprising that embryos nullizygous for Rb show phenotypic abnormalities largely restricted to the nervous system, lens and hematopoietic system (table 1). Additionally, p107/p130 nullizygous mice develop specific abnormalities in endochondral bone formation. These mouse models demonstrate that pocket proteins have cell-lineage-specific functions that are important during development.

A universal role for Rb in tumor suppression has also been implicated by the frequent inactivation of the cell cycle regulatory pathway centered on Rb in most human cancers. Challenging a ubiquitous role for Rb in tumorigenesis, however, is the fact that humans with germline Rb mutations are at increased risk for developing distinct tumors including retinoblastoma, sarcoma, melanoma and lung cancer [50–53]. Interestingly, Rb gene mutations are frequently observed in only a very small subset of sporadic human malignancies, and that list also includes retinoblastoma and small-cell lung cancer [6]. $Rb^{+/-}$ mice develop tumors restricted to the pituitary, thyroid and adrenal glands (table 2). These findings in mouse models along with the clinicopathologic data in humans provide evidence that certain cell types are exquisitely sensitive to Rb loss, and suggest that Rb has cell-specific functions important in tumor suppression as well as development.

How does loss of Rb function lead to different effects in different cell types? Moreover, why are specific lineages exquisitely sensitive to Rb-mediated tumor suppression? Epigenetic features and cellular context likely play a role in how a cell responds to loss of Rb function. Non-genetic cellular properties that may dictate susceptibility to Rb loss are not limited to DNA methylation and histone modifications, but could also include differences in signaling pathways that control cell fate commitment, developmental-stage-specific expression of cell cycle regulatory factors and cellular microenvironment. Defining the cell of origin for malignancies resulting from Rb deficiency is a first step in elucidating the importance of cellular context in tumor initiation and progression.

The retina has been used as a model system to study the role of cellular context in tumor formation. The retina is unique in that Rb mutations are known to be the initiating genetic lesion in the development of retinoblastoma. Additionally, retinal development is well characterized and tools are available to distinguish and manipulate distinct cell types *in vivo*. Recent studies in genetically engineered mice have implicated postmitotic lineage-specific transitional cells as the cells of origin for retinoblastoma [54]. These cells appear to have unique innate features which predispose them to forming tumors. Unlike the situation in humans, Rb ablation alone in the mouse does not lead to retinoblastoma. However, conditional Rb ablation in the retina of p107 null mice results in the development of retinoblastoma (table 3). Interestingly, Rb/p107 double-null retinal transitional cells destined to amacrine, horizontal and Muller cell fates were intrinsically resistant to the apoptosis that resulted from loss of

Rb family function in other cell types. Instead, these cells survived, underwent a finite period of ectopic proliferation, and then exited the cell cycle by an Rb-independent mechanism linked to terminal differentiation. These findings lead the authors to propose a 'differentiation model' to explain tumor development. According to this model, loss of Rb function results in an extended, but finite, period of ectopic proliferation in specific transitional cell types intrinsically resistant to the cell death induced by loss of Rb function. Subsequent mutations are then required to overcome the growth arrest associated with terminal differentiation.

Increased resistance to apoptosis is a hallmark of tumor cells usually believed to be acquired through mutation of genes such as p53. Intrinsic death resistance in the cells of origin for retinoblastoma, and possibly other Rb-dependent tumors, would reduce the number of genetic alterations required for transformation. This could explain why distinct cell types more readily develop tumors after loss of Rb function, and why retinoblastomas are believed to arise after fewer genetic alterations relative to other malignancies.

Pocket proteins have unique and overlapping functions

Cellular sensitivity to Rb deficiency may reflect differing degrees of functional compensation or redundancy among family members in distinct cell types. Rb, p107 and p130 share structural homology (fig. 2) and have overlapping expression patterns during development [47]. Additionally, all three pocket proteins inhibit E2F-responsive promoters, recruit chromatin-remodeling enzymes, actively repress transcription and growth arrest cells when overexpressed [12, 16]. An important distinction among the pocket proteins, however, is that Rb, but not p107 and p130, has been shown to be a bona fide tumor suppressor in humans [28]. Defining the overlapping versus distinct functions of Rb family proteins is therefore an important step to understanding the unique role of Rb in tumor suppression.

Rb, but not p107 and p130, is essential for development (table 1). Rb nullizygous mice die during mid-gestation with defects in the nervous system, hematopoietic system and lens. In contrast, p107 and p130 nullizygous mice develop normally in the same genetic background. Of note, p130 null mice show embryonic lethality and p107 deficiency results in impaired growth and a myeloproliferative disorder in the Balb/cJ background, suggesting that strain-specific modifiers may play a role in determining phenotypic outcomes. Mice nullizygous for both p130 and p107 die at birth with abnormalities in endochondral bone formation. Distinct phenotypes in mice with ablation of specific pocket protein members demonstrate that these proteins have unique functions during development.

Mouse models also provide evidence that pocket proteins have redundant or compensatory functions (table 1). p107 and p130 are capable of partially compensating for Rb deficiency during development. Embryos nullizygous for p107 or p130 in conjunction with Rb die approximately 2 days earlier than Rb null embryos and show more severe defects in the nervous and hematopoietic systems. Additionally, p130 is capable of compensating for Rb deficiency in cardiac muscle development [55] and p107 can compensate for loss of Rb function in the epidermis [40]. p130 and p107 also have overlapping functions, since p107/p130 double-knockout mice die at birth with aberrant bone and epidermal development, whereas single-knockout mice develop normally.

Pocket proteins have unique and overlapping functions in tumorigenesis as well as in development. Rb heterozygous mice are prone to develop tumors of the pituitary, thyroid and adrenal glands (table 2). p107 and p130 ablation alone or in combination does not predispose to tumors. However, p107 and p130 can function to suppress tumorigenesis in the context of Rb deficiency. Mice nullizygous for Rb do not develop retinoblastomas as is seen in humans. However, loss of p107 or p130 in combination with Rb results in retinoblastoma (table 1). Additionally, Rb ablation in astrocytes [56] and mammary epithelial cells [57] results in no phenotypic abnormalities, whereas loss of total pocket protein function by expression of a truncated form of SV40 large T antigen leads to tumor formation [58, 59]. Furthermore, chimeric Rb/p107 and Rb/p130 null mice develop tumors in addition to those seen with Rb ablation alone [60]. The tumor spectra in Rb-, Rb/p107- and Rb/p130-deficient mice do not totally overlap, providing evidence that the pocket proteins have unique as well as overlapping functions in tumor suppression.

Timing of Rb ablation is critical to phenotypic outcomes in cells in culture

Germline inactivation of Rb family proteins has provided great insight into the role of pocket proteins in development and tumor suppression. Furthermore, combined inactivation of pocket proteins along with other genes has provided insights into the molecular pathways underlying Rb-family-mediated regulation of apoptosis, proliferation and differentiation. One limitation of germline inactivation, however, is that germline mutations may not mimic somatic mutations that occur in sporadic human malignancies. Conditional gene ablation using the Cre-loxP and Flp-frt site-specific DNA recombination systems allows for tissue-specific ablation *in vivo*. Additionally, these strategies allow for acute ablation in cells in

culture and, when combined with inducible gene induction, temporally controlled gene ablation *in vivo*.

Timing of Rb ablation has been shown to be critical to phenotypic outcomes in embryonic fibroblasts and epidermal cells in culture. Sage et al. [61] demonstrated that acute loss of Rb function in MEFs in culture has phenotypic consequences different from germline loss of Rb function. Specifically, acute loss of Rb in quiescent and senescent cells was sufficient for cell cycle re-entry whereas cells undergoing germline Rb loss did not show this phenotype. The difference in phenotypic outcomes is explained at least in part by functional compensation by p107 in the case of germline, but not acute, Rb inactivation. Ruiz et al. [40] have also shown that keratinocytes with acute loss of Rb are refractory to cell cycle arrest upon calcium-induced differentiation whereas cultured keratinocytes that have undergone loss of Rb *in vivo* responded similar to wild-type cells. These results demonstrate that timing of Rb ablation is critical to phenotypic outcomes in cultured cells. Furthermore, the studies show that functional compensation by p107 occurs during development but is not immediately installed after acute loss of Rb function in cells in culture. Inducible conditional knockout systems in mice are now feasible and will be instrumental in determining whether the timing of Rb ablation has physiological relevance in the context of cell function *in vivo*.

Rb functions through both cell-autonomous and cell-non-autonomous mechanisms

The generation of chimeric mice provided the first indication that Rb had cell-non-autonomous functions. Rb null embryonic cells were capable of contributing to most adult tissues including those organs that showed marked phenotypic abnormalities in Rb-ablated embryos [62, 63]. Central nervous system abnormalities were not detected in chimeric mice despite an 80% contribution by Rb null cells. Additionally, phenotypically normal peripheral blood erythrocytes were shown to be nullizygous for Rb, and, conversely, abnormal nucleated erythrocytes were wild-type for Rb. This discordance between cellular phenotype and genotype provided evidence that the neural and hematopoietic defects in germline Rb knockout mice were, at least in part, due to cell-non-autonomous Rb functions.

Mechanisms accounting for the cell-non-autonomous roles of Rb were recently uncovered by studies directly demonstrating that many of the defects seen in Rb null embryos are secondary to inadequate placental function. Wu et al. [43] created Rb-deficient embryos supplied by a wild-type placenta using conditional knockout strategies and tetraploid aggregation. These Rb-deficient pups survived to term and lacked many of the neurological and erythroid abnormalities seen in conventional Rb knockout mice. Consistent with these results, Rb ablation restricted to the nervous system did not result in the aberrant neuronal apoptosis seen in Rb-deficient embryos [44].

The defective erythropoiesis present in Rb null embryos was recently shown to be secondary to profound abnormalities in fetal liver macrophages rather than an intrinsic defect in erythrocytes. Iavarone et al. [64] demonstrated that Rb promotes differentiation of macrophages by opposing the inhibitory functions of Id2 on a transcription factor required for macrophage differentiation. Id2 is a helix-loop-helix protein capable of physically interacting with the active, hypophosphorylated form of Rb. The aberrant erythropoiesis seen in Rb null embryos results from unrestrained Id2 activity in macrophages. Consistent with this mechanism, Id2 deficiency rescues the erythropoietic defect in Rb knockout mice [65]. Taken together, these recent findings in mouse models identify critical functions for Rb in extra-embryonic tissues and fetal macrophages, and provide mechanisms for the cell-non-autonomous roles of Rb during development.

Rb also functions in a cell-autonomous manner. Despite the increased survival of Rb null pups rescued by wild-type placentas and Id2, these mice still die at birth and show severe skeletal muscle abnormalities [42, 43, 65]. Additionally, the ocular lens in rescued Rb null pups shows ectopic proliferation associated with apoptosis similar to that found in conventional Rb null embryos. Rb chimeric mice also display tissue-specific developmental abnormalities and tumors that represent cell-autonomous Rb functions [62, 63]. Ectopic mitoses and substantial cell degeneration were noted in the retina, and cataracts developed secondary to aberrant formation of the ocular lens. Chimeric Rb mice also consistently developed pituitary tumors and adrenal hyperplasia similar to that seen in $Rb^{+/-}$ mice. The tumors were exclusively derived from Rb null cells providing evidence that loss of Rb function was required for malignant transformation. The cell-autonomous nature of the tumor phenotype is further supported by the fact that pituitary tumors in $Rb^{+/-}$ mice consistently show loss of the wild-type Rb allele [66–68], chimeric mice develop tumors at an accelerated rate compared to $Rb^{+/-}$ mice [62] and conditional Rb ablation in the pituitary results in tumors [69]. Thus, Rb null mice manifest abnormalities resulting from loss of these cell-autonomous functions in conjunction with phenotypes that are cell-non-autonomous in nature.

Molecular pathways underlying Rb-family mediated functions

Significant insights into the molecular mechanisms underlying Rb-family-mediated regulation have been gained

by crossing pocket-protein-deficient mice into various different genetic backgrounds (tables 1, 2). Rb ablation during development results in apoptosis that occurs through different molecular pathways in distinct tissue types [21]. Apoptosis occurs through a p53-dependent mechanism in the central nervous system and lens. In contrast, apoptosis in the peripheral nervous system is p53 independent. Additionally, apoptosis in the peripheral nervous system is mediated through caspase 3 whereas the central nervous system requires Apafl but not caspase 3.

Rb-E2F complexes are capable of binding DNA and repressing transcription of genes important in cell cycle regulation. Accordingly, deregulated E2F activity plays a role in the ectopic proliferation in Rb mutant mice. Compound Rb and E2F1, E2F2 or E2F3 nullizygous mice survive longer and show less aberrant proliferation compared to Rb null embryos (table 1). Additionally, ablation of E2F1 and E2F3 partially rescues the apoptosis and defective erythropoiesis in Rb null embryos. Studies such as these are important in determining the *in vivo* biologic functions of Rb that are likely to be mediated by E2F-dependent gene regulation.

The E2F- and p53-dependent apoptosis seen in Rb null embryos suggested p19ARF as a mediator in this phenotype. p19ARF is a transcriptional target of E2F1 and regulates p53 activity by binding to Mdm2 and blocking Mdm2-induced p53 degradation [70, 71]. Mice nullizygous for p19ARF develop malignancies, supporting a tumor-suppressive role for this protein. In addition, p19ARF is the product of an alternative transcript within the p16 gene, and deletions of the INK4a/ARF locus have been detected in a variety of human tumors. Surprisingly, p19ARF was not required for the apoptosis seen in Rb mutant mouse embryos [72]. Moreover, p19ARF deficiency actually exacerbated the excessive proliferation and apoptosis in the peripheral nervous system of Rb null mice. p19ARF is also dispensable for the p53/E2F1-mediated apoptosis seen in tumors resulting from loss of total pocket protein function [73]. Thus, although p19ARF functions to transmit a signal from E2F1 to p53, it is dispensable for oncogenic-stress-induced apoptosis and tumor suppression *in vivo*.

Mouse models support common mechanisms for tumor initiation and progression in divergent cell types. Total pocket protein inactivation using a truncated SV40 large T antigen that lacks the domain required for p53 inactivation results in tumors in choroid plexus epithelium, mammary epithelial cells and astrocytes [58, 59, 74]. Inactivating these critical cell cycle regulators leads to increased cell proliferation and apoptosis. In the brain and mammary epithelium, apoptosis and inhibition of tumor growth are mediated through p53. In the lung epithelium, Rb ablation alone does not result in tumors; however, somatic ablation of Rb in conjunction with p53 leads to development of small-cell lung cancer (SCLC) [75, 76]. These tumors share morphologic features and a neuroendocrine phenotype with human SCLC. Lung epithelial ablation of Rb during development resulted in hypercellular neuroendocrine lesions that may represent precursors to SCLC; however, these lesions have not been reported to go on to form tumors [75]. This suggests that Rb is required, but not sufficient, for the generation of SCLC, and that loss of p53 provides an additional requirement for tumorigenesis. Interestingly, Rb and p53 mutations are frequently detected in human SCLC [77–79] and therefore the mouse model is likely mimicking the carcinogenic process that occurs in humans.

Loss of p53 function accelerates tumor growth by diminishing the apoptosis induced by pocket protein inactivation. E2F1 signals the p53-dependent apoptosis but, unlike p53, E2F1 deficiency does not accelerate tumor growth [80]. This can be explained by the finding that E2F1 deficiency also impairs cell proliferation. Thus, in the case of E2F1 deficiency, impaired proliferation counterbalances the effect of apoptosis reduction. Importantly, p53 loss can also facilitate epithelial tumor progression by mechanisms distinct from apoptosis reduction [81]. p53 deficiency has been associated with chromosomal instability; however, chromosomal instability does not account for epithelial tumor progression in Rb-family-deficient choroid plexus epithelium. This raises the possibility that other p53 functions may be important in suppressing tumor progression.

The apoptotic response to pocket protein deficiency is not universally dependent on p53. Inactivation of Rb family proteins in astrocytes predisposes mice to malignant astrocytomas [59]. p53 haplodeficiency had no effect on the development of these tumors. In contrast, apoptosis and tumor suppression was mediated through PTEN. Accordingly, PTEN haplodeficiency resulted in accelerated tumor progression and reduced apoptosis. Therefore, a common theme emerges once again: the cellular response to Rb family ablation is context specific.

Remaining questions

Research on the Rb family proteins has resulted in tremendous progress over the last two decades. Novel, and often surprising, insights into Rb family function have been gained through the analysis of genetically engineered mice. These studies have also highlighted important gaps in our knowledge of how pocket proteins function *in vivo*. Many of the Rb-family-dependent phenotypes *in vivo* cannot be fully explained by our current molecular understanding of pocket protein function. Some questions that remain include: (i) why are different components of the Rb pathway mutated in specific

tumor types, (ii) why does Rb deficiency lead to cell-type-specific abnormalities and (iii) why is Rb, but not p130 and p107, a bona fide tumor suppressor? It appears that despite our tremendous advances, the essential biologic functions of Rb that are required for proper development and tumor suppression are not clearly defined. What is clear from the *in vivo* studies, however, is that pocket proteins have overlapping and unique functions, and that their roles in development and tumor suppression are context specific. A major challenge for the future is to bridge the gap that currently exists between our molecular understanding of Rb function and pocket-protein-dependent phenotypes *in vivo*. While this remains a formidable task, it is clear that Rb has provided us with an exciting and informative journey thus far, and there is reason to believe that exciting discoveries still await us.

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