Insights from Animal Models on the Origins and Progression of Retinoblastoma

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Abstract: The ***RB*** gene was discovered 20 years ago because of its role in the childhood eye cancer retinoblastoma. However, surprisingly little progress was made in defining the role of RB protein in the retina. In the last two years, new models exploiting conditional deletion of the mouse ***Rb*** gene have altered this picture radically. These models provide insight into the first Rb function, the cell of origin of retinoblastoma, the window during which Rb acts, distinct cell-specific defenses against ***Rb*** loss, the number and type of post-***Rb*** lesions required for transformation, why pediatric tumors exist, the controversial role of the p53 pathway in retinoblastoma, and the reason why the disease is virtually unique to humans. Two years have dramatically improved our understanding of Rb function in the tissue that gave us this important tumor suppressor.

INTRODUCTION

Retinoblastoma arises in 1 in 20 000 children, yet despite its rarity, it has unlocked some of the most valuable secrets of neoplasia [1]. In 1971 Knudson used patient data to propose that retinoblastoma requires two rate-limiting events, the classic "two-hit hypothesis". Later, it was realized that both hits likely target the same locus, and by 1983 Cavenee ***et al.*** had used restriction fragment length polymorphisms to detail, for the first time, the chromosomal events that can lead to loss of both copies of a tumor suppressor [2, 3]. Three years later, and 27 years after Harris ***et al.*** used cell fusion assays to reveal their existence [4], the first tumor suppressor, ***RB***, was isolated [5]. Subsequently ***RB*** mutations were linked to many cancers, and eventually it was realized that this gene was at the heart of a pathway implicated in most forms of cancer [6]. In 1988 Whyte ***et al.*** showed that RB protein binds the adenoviral protein E1A, the first clear indication that tumor suppressors and oncoproteins interact directly (p53-large T antigen binding was known, but the tumor suppressor status of p53 was under debate) [7]. These and other subsequent fundamental contributions transformed our understanding of cancer. Despite these advances, there has been scant insight into RB function in the tissue that led to its discovery. In the last two years, however, conditional ***Rb*** deletion models in mouse retina have revealed intriguing insights into Rb protein function in retinal development, and several likely have broad applicability to cancer.

An important feature of retinoblastoma is that it is one of the few cancers for which we know the initiating event. Cellular context determines sensitivity to specific oncogenic insults, yet we know little about any cell-of origin and why it is tumor prone. Because the starting point of retinoblastoma is ***RB*** mutation, and the gene is so important in cancer, this rare tumor can serve as an ideal model to probe the step-wise evolution of a cancer from a single cell to an aggressive tumor. Animal models are critical for such studies, and we review their development here. We begin with basic information on human retinoblastoma, essential background on RB function, and an overview of retinal development.

HUMAN RETINOBLASTOMA – MORE THAN JUST TWO HITS

Retinoblastoma is the most common childhood ocular malignancy. About 40% of cases are inherited, while the others arise from spontaneous somatic mutations. In heritable cases, the first hit is in every cell and a second somatic hit is almost inevitable, ensuring the development of multiple bilateral, retinal tumors. The non-inherited form of the disease requires two somatic mutations, which is rare, so these cancers are typically unilateral and unifocal. Some non-inherited cases of retinoblastoma are bilateral due to early appearance of a somatic mutation in development resulting in mosaicism, and some inherited cases of retinoblastoma are unilateral due to low penetrance mutations that partially disrupt RB protein function (see [1] for details).

It is well known that loss of both ***RB*** alleles is critical for retinoblastoma development, but it is less well appreciated that almost every tumor has additional genetic lesions. Karyotyping, comparative genomic hybridization (CGH) and quantitative multiplex PCR (QMPCR) revealed high frequency gains or losses at several loci. Of 162 tumors

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analyzed in five CGH studies, the three most frequent changes observed were gain of 6p22 (53%) or 1q31-32 (50%), and loss of 16q (30%) [8-12]. Other changes are seen at lower frequencies, such as gain of 2p24-25 (17%), 13q32-24 (8%) and 19 (8%). QM-PCR analysis provides better resolution than CGH and analysis at the three most commonly altered regions revealed an even higher fraction of changes. Thus, 6p22 gains were seen in 59% (86/146) of tumors [13, 14], 1q31-32 gains were detected in 71% (39/55) of tumors [15], and 16q22 loss was observed in 54% (22/41) of tumors [16].

Genomic stability in retinoblastoma appears to be variable and correlates with specific events. In one study, 1q, 6p or 2p gains were associated with a higher frequency of other aberrations [11]. However, others saw similar frequencies of abnormalities (3.6-4.4 per case) in the presence or absence of 6p gain [10].

Recently, genes have been identified at commonly altered loci that may provide a selective advantage for retinoblastoma cells. A promising candidate to explain 6p22 gain was ***KIF13A*** (***RBKIN***), a kinesin family member, and antisense oligonucleotides to this target had a modest negative effect on the growth of two retinoblastoma cell lines [13]. However, subsequent analyses suggested a better correlation with ***E2F3*** and ***DEK*** expression at this locus [14, 17]. Another kinesin, ***KIF14A***, has been implicated as the critical gene at the 1q31-32 locus, and is frequently over expressed in tumors other than retinoblastoma [15]. At the 16q22 locus, loss of expression of ***cadherin 11*** (***CAD11***), but not ***cadherin 13*** (***CAD13***) or other genes, was linked to retinoblastoma progression [16]. These are potentially exciting advances and animal models will be indispensable in testing whether, when, and how these candidates influence the progression of retinoblastoma.

Gain of 1q and loss of 16q often occur together [11, 12], implying cooperation between genes at different loci. Intriguingly, tumors removed from older children typically have more alterations than those from younger children [8, 9, 12]. Late diagnosed tumors could either reflect slow growing cells, fast growing cells that arise from a slow growing tumor, or fast growing cells that arose from transformation of a late-born retinal cell type. In any case, these data suggest that some tumors may require more post-***RB*** events than others. Later, we discuss in more detail the idea that the cell of origin influences the number and type of post-***RB*** events required for malignancy.

A KEY Rb FUNCTION *IN VIVO* – PERMANENT CELL CYCLE EXIT

RB is a well-established negative regulator of the cell cycle [6, 18]. We will briefly review how RB participates in this cycle, re-emphasize important modifications to the original model discussed in several other recent reviews [18-20], then end by emphasizing the major role RB plays in coupling terminal differentiation to permanent cell cycle exit ***in vivo***.

In the early 90's several groups showed that ectopic expression of RB in many (but not all) cell types can increase the fraction of cells in G1 [21-23]. This effect fitted the observation that as cells proceed from G1 to M phase, Rb is gradually hyperphosphorylated by CDK/Cyclin complexes that promote cell cycle progression (reviewed in [1, 6, 18]). It also explained why viral oncoproteins such as Simian virus 40 (SV40) large T, human papilloma virus (HPV) E7, and adenovirus E1A all bind hypophosphorylated RB [7, 24-28]. RB is part of a protein family that includes the closely related p107 and p130 proteins, and these proteins mimic many RB activities [29, 30]. Subsequently, it was shown that RB family proteins bind and inhibit transactivation by E2F, a family of proteins required for induction of genes critical for cell cycle progression ([31-34], reviewed in [35]). These discoveries cemented the view that RB tempers the pace of cycling cells by blocking expression of E2F targets. Yet, as discussed below, this simple model did not fit subsequent discoveries.

The glorified "Rb is all" view of cell cycle regulation was modified by studies on mouse embryo fibroblasts (MEFs) from knockout mice. In ***Rb***$^{-/-}$ primary or immortalized MEFs G1 phase is shorter and cells proceed from serum-starved quiescence more quickly through G1 into S-phase [36-38]. Loss of ***p107*** or ***p130*** alone does not affect these parameters, but deleting both has a similar effect as ***Rb*** loss on passage through G1 [38-40]. However, despite the shorter G1, overall rates of cell division in ***Rb***$^{-/-}$ and ***p107***$^{-/-}$***;p130***$^{-/-}$ MEFs are virtually identical to wild type due to a lengthened S-phase [36, 37]. The probable (yet surprising at the time) answer to this paradox is that in cycling MEFs, Rb and p107/p130 regulate distinct subsets of cell cycle genes [37, 38, 41-43]. It is believed that deregulating either set accelerates passage through G1, but the lag in other gene induction events slows passage through S-phase, so the collective effect on cell cycle length is marginal. In support of this model, when all three ***Rb***-related genes were deleted, triple knockout (TKO) MEFs did (finally!) divide more rapidly than wild type (WT) or double knockout (DKO) MEFs [44, 45].

Such ***in vitro*** studies have revealed important fundamental insight into the role of pocket proteins in cell cycle regulation and the targets that mediate this function. Yet deleting ***Rb*** alone has a far more dramatic effect on division in a mouse than in MEFs. ***Rb*** absence is lethal by E13-15 and is associated with extensive ectopic division and apoptosis [46-48]. As we will discuss below, apoptosis in several tissues is an indirect effect of hypoxia related to placental defects, but the aberrant ectopic division is cell autonomous (a direct effect of Rb loss on the affected cell). Clearly, Rb has a vital role in cell

division ***in vivo*** that is not completely rescued by p107 or p130. Careful analysis in several tissues has revealed that this role is in permanent cell cycle exit during terminal differentiation. For example, when dividing lens epithelia migrate inwards and differentiate into lens fibers, this event is normally coupled to cell cycle exit, but in the absence of Rb differentiating fibers continue to divide [49]. Similarly, Rb is also essential for permanent cell cycle exit when myoblasts switch to myocytes and when neural progenitors on the ventricular surfaces of the brain migrate inwards and begin differentiating into neurons [50-52]. Rb absence does not interfere with the initial differentiation steps. For example, ***Rb$^{-/-}$*** fibers migrate properly and express α-crystallin (an early marker of differentiation) and newborn ***Rb$^{-/-}$*** neurons in the forebrain also migrate away from the ventricular surface and express β-III tubulin (an early marker of neurogenesis) [49, 51]. Much of the ectopic division in the Rb$^{-/-}$ CNS or lens is rescued in the absence of E2F1 [53]. These data complement ***in vitro*** studies in a granulocyte model in which retinoic acid induced HL60 differentiation triggers RB dephosphorylation and binding to E2Fs [54]. Thus, a major function of Rb ***in vivo*** is to quench E2F activity in terminally differentiating cells, locking them out of the cell cycle. Not surprisingly, therefore, this is also the case in the developing retina (see below).

RETINAL DEVELOPMENT – THE BASICS

A brief overview of retinal development will facilitate subsequent discussion of the effects of Rb loss. Retinogenesis has three overlapping steps (Fig. **1A**):

1. Multiplication of Retinal Progenitor Cells (RPCs)

In the rat, retinal cell number increases 40-fold between embryonic day (E) 14 (6.2×10^4 cells) and post-natal day (P) 8 (2.5×10^7 cells) [55]. An intriguing property of RPCs is that their nuclei migrate along vertical processes attached to the outer and inner retinal surfaces according to their position in the cell cycle. M-phase nuclei lie on the outermost (ventricular/apical) surface, S-phase nuclei lie anywhere in the inner half of the retina depending on which stage of S-phase they have reached, and G1 and G2 nuclei lie between S and M phase nuclei. At M-phase, RPCs choose whether to generate two more RPCs, one RPC and a post-mitotic retinal transition cell (RTC)*, or two RTCs. The latter becomes more likely as development proceeds. The position of mitotic RPCs on the outer surface is a useful property to distinguish normal dividing RPCs from abnormally mitotic RTCs, most of which would be located in different regions of the inner retina. (*Note that several groups, including our own, have used “precursors” rather than “RTCs” to describe differentiating cells. However the term “precursor” can be confusing as it is also used to describe dividing progenitors in other brain structures).

2. Birth of Post-Mitotic Retinal Transition Cells

Once born, RTCs migrate inward to their final positions in the mature retina (except for a small percentage of cells that will become cones, which stay on the outer surface). RTC birth involves two events: (i) differentiation (evident from induction of differentiation markers and migration to a final position in the retina) and (ii) cell cycle exit. As we will detail later, there is convincing evidence that the critical role for Rb in retinal development is in cell cycle exit. Differentiation of RPCs into RTCs is Rb-independent. Abnormal division of differentiating Rb-deficient RTCs provides a window of opportunity for other mutations and full neoplastic transformation.

3. Terminal Differentiation of RTCs into Six Neurons and One Type of Glia

Mature retinal cells are organized into three layers. The outer nuclear layer (ONL) contains rod and cone cell bodies (with abundant rod nuclei making up most of the layer and cone nuclei the outermost level of the ONL), the middle or inner nuclear layer (INL) contains horizontal, bipolar, and amacrine interneuron cell bodies as well as Müller glia cell bodies, and the innermost ganglion cell layer (GCL) contains a 1:1 mix of ganglion and displaced amacrine cell bodies. Ganglion cell axons project to the brain ***via*** the optic nerve. RTCs that give rise to different neurons and glia are born at specific times in development. In rodents, ganglion cells, cone photoreceptors, horizontal interneurons and the majority of amacrine interneurons are born pre-nataly, while bipolar interneurons and Müller glia are born post-nataly (Fig. **1B**). Rod photoreceptors are born both before and after partition (Fig. **1B**).

A fourth component of retinogenesis affects a small fraction of retinal cells, such as blood vessels, astrocytes and microglia. These cells are not derived from RPCs but migrate into the retina after partition [56].

ANIMAL MODELS – THE EARLY DAYS (SEE TABLE 1)

In the 1890s light microscopy studies by Flexner and Wintersteiner suggested that photoreceptors might give rise to retinal tumors based on the morphology of rosettes observed in the tumor samples [57, 58]. Later observation of emerging human tumors suggested that tumors may arise from the inner nuclear layer (INL) of the retina [59], which coincides with the origin of retinoblastoma in numerous animal models [60-63]. However, early samples of human tumors are rare, making it difficult to use the human disease to elucidate exact origin. Thus, animal models are necessary to provide clues about the origin and progression of retinoblastoma as well as to provide tools for development of treatments. Generating these models has proven challenging as spontaneous retinoblastoma appears to be almost exclusively a human disease.

Retinoblastoma has almost never been observed in other species, with the exception of very rare cases in dogs and one case in llama [64-66]. Animal models of retinoblastoma have provided insight into this species-specific difference (see below).

In the 1940's, the first attempts to induce spontaneous retinal tumor growth by injecting carcinogens into the rat eyes were largely unsuccessful [67, 68]. In the 1960's, advances in viral oncology were exploited and revealed that adenovirus 12 (Ad-12) consistently produced neurogenic tumors in rodent laboratory animals [69-71]. Subsequently, intraocular injection of Ad-12 produced retinal tumors in young rats and mice [72-76] as well as baboons [77], and a hamster model was generated using JC papovavirus [78]. In general, tumors appeared with variable frequency, depending on the model and conditions, displaying poorly differentiated rosettes and no intracranial metastasis. These findings pre-dated the realization that viral oncoproteins sequester Rb, its relatives p107 and p130, and many other proteins that may promote transformation (see below). With the benefit of hindsight, these assays support the idea that non-human animals resist retinoblastoma with the help of p107 and/or p130.

Concurrently with DNA tumor virus studies, several groups noted successful tumor growth after intra-ocular implantation of primary human retinoblastoma or cell lines into either rabbits [79] or rodents [80-83].

Table 1. Early Non-Transgenic Models

CARCINOGEN INJECTION AND IMPLANTATION INTO THE EYE			
CHEMICAL	**ANIMALS**	**NOTES**	**REFERENCES**
Dibenzanthracene, superheated oil	adult rats	Gliosis and folding of the retina; probably not malignant.	[67]
Methylcholanthrene	adult mice	Tumors of ocular tissues observed but no retinoblastoma.	[68]
RETINAL CELLS TRANSFORMED *IN VITRO* WITH DNA TUMOR VIRUS			
VIRUS	**ANIMALS**	**NOTES**	**REFERENCES**
Ad-12	young adult hamsters	Transfected cells form tumors when injected s.c. into irradiated syngeneic recipients.	[211]
INTRAOCULAR INJECTION OF DNA TUMOR VIRUS			
VIRUS	**ANIMALS**	**NOTES**	**REFERENCES**
Ad-12	newborn or several days old rats/mice	Tumors occur with variable frequency (5-16 % of animals) depending on conditions within several months, displaying poorly differentiated rosettes.	[71-76]
JC papovavirus	newborn hamsters	Invasion of the optic nerve observed but no intra-cranial metastasis.	[78]
Ad-12	new born baboons	3/21 animals develop tumors within 1yr. FW rosettes observed.	[77]
INTRAOCULAR INJECTION OF RETINOBLASTOMA CELL LINES OR PRIMARY RETINOBLASTOMA			
	ANIMALS/ INJECTION SITE	**NOTES**	**REFERENCES**
Human cell lines			
Y-79/WERI-Rb-1	rabbits/a.c.	In all cases solid tumors form, displaying undifferentiated characteristics, never forming rosettes.	[79]
Y-79	nude mice/a.c.		[80]
Y-79/WERI-Rb-1	Rag-2 mice/v.c.		[82]
Y-79	rats/s.r.		[83]
Human tissue	nude mice/a.c	Tumor histology reflects the original tumor (e.g., rosettes observed when also observed in the original tumor).	[80, 81]
Mouse Ad-12 induced:			
tissue	mice/v.c	Tumor histology reflects the original tumors.	[84]
cell line (EXP-5)	rats/v.c		[85]

a.c.: anterior chamber; v.c.: vitreous chamber; s.r.: sub-retinal space; FW: Flexner-Wintersteiner.

Ad-12 induced mouse tumor tissue or rat tumor-derived cell lines have also been injected into the vitreous of mice or rats, and the pathology of the tumors generated in this way mimics the tumors generated by injection of Ad-12 alone [74, 84, 85]. Studies using multiply-passaged cell lines and tissue samples from mature tumors do not reveal much about the origin or progression of the disease. These models can serve as therapeutic tools, but they may not reveal how younger tumors in human tissue would respond to a given drug.

The early attempts to generate retinoblastoma with viruses and cell lines were followed initially with transgenic mice expressing viral oncoproteins, then with germ line knockouts, and most recently with conditional knockout (CKO) approaches. Below, we discuss these genetic models in sequence then summarize several new insights and questions raised by CKO models.

TRANSGENIC VIRAL ONCOPROTEIN MODELS (SEE TABLE 2)

The earliest inheritable models of retinoblastoma exploited viral oncoprotein expression in the developing mouse retina. Below, we outline the models, but begin by summarizing the numerous factors targeted by these viral oncoproteins.

Protein Targets of Viral Oncoproteins

There is a commonly held but erroneous assumption that viral oncoprotein expression is equivalent to inactivating Rb family members. In fact, there are multiple protein targets. Importantly, no studies have attempted to elucidate the role of most of these interactions in retinoblastoma models.

SV40 large T antigen is a 708 amino acid protein with multiple interactions surface several of which, in addition to Rb binding, are implicated in transformation (reviewed in [86]). Like other viral proteins, large T utilizes an LxCxE motif to interact with the A-B pocket domain of RB. The other well known large T partner is the tumor suppressor p53, yet while these two interactions are critical for transformation, they are not sufficient [87-89]. In addition to these proteins, large T also binds the chaperone hsc70, the cullin Cul7, which is part of an E3 ligase complex involved in ubiquitin-mediated protein degradation [90], Fbw7, also involved in ubiquitination [91], the transcription elongation factor TEF-1 [92], the mitotic spindle protein Bub1 [93], and Nbs1, which is part of the MRN complex involved in DNA repair [94]. Several of these interactions are important for large T effects. For example Cul7 binding is required for anchorage independent growth in mouse embryonic fibroblasts (MEFs) [90], and Bub1 binding is required for focus formation in Rat-1 cells [93]. Moreover, large T could perturb

Table 2. DNA Tumors Virus Proteins

Promoter/ Onset of expression in the retina	Location of viral oncoprotein expression	Tumors	Time of tumors observed/ penetrance	References
SV-40 T/t				
LH-β not reported	INL midbrain	retina: INL, bilateral, multi-focal, FW&HW rosettes midbrain (PNET)	2 mo 100% 27%	[124, 134, 212, 213]
PNMT P3	retina: amacrine and horizontal cells adrenal medulla	peripheral retina: INL/GCL amacrine/horizontal cell markers phaeochromocytoma	9 wks 100%	[127, 128]
IRBP E13	retina: rod/cone RTCs pineal gland	retina: ONL, bilateral, HW rosettes Midbrain (PNET), Pineal gland	2 wks 100% 100%	[129, 130]
Rhodopsin P1	retina: rods pineal gland	no retinal tumors Dissociated and s.c. injected retinas form tumors in nude mice. pineal gland	 45 d 5/6 mice	[131, 214]
HPV-16 E6 and E7				
αA-crystallin not reported	retina in one transgenic line and lens	retina: INL, focal, HW rosettes; invades brain, metastasizes to cervical lymph nodes	>3 mo (bckd dependent)	[138]
HPV- 16 E7				
IRBP E14	retina: rod/cone RTCs pineal gland	no retinal tumors pineal gland	Several wks	[133]
***p53*$^{-/-}$ bckd**		retina: ONL, bilateral pineal gland		

Bckd: background; d: day; HW: Homer-Wright; INL: inner nuclear layer; mo: month; ONL: outer nuclear layer; PNET: primitive neuron-ectodermal tumor; RTC: retinal transition cell; s.c.: sub-cutaneously; wk: week

genomic stability through its interaction with p53, Bub1 and/or Nbs1, promoting mutations that favor transformation.

Many of the SV40 large T transgenic models, including all those involving retinal expression, do not exploit a large T cDNA, but instead use the early region of the SV40 genome, which expresses both **small t** and large T antigens. This point is usually overlooked when animal models of retinoblastoma are discussed. Small t is a 174-residue protein that shares the same N-terminus as large T, but continued translation of the large T intron generates a different, shorter C-terminus that binds protein phosphatase 2a (PP2A). Small t is essential for transformation in multiple contexts and interaction with PP2A is critical for this activity [95, 96].

HPV E7 is a 98-residue protein with three conserved regions (CR1-3). The LxCxE motif necessary for binding RB family proteins is present in CR2. E7 triggers RB degradation and other regions in the N-terminus are required for this effect [97-100]. E7 has similar effects on p107 and p130 [100]. In addition to the RB family, E7 binds several other proteins including Mi2β, which in turn allows it to indirectly bind histone deacetylases (HDACs) [101, 102]. E7 also interacts with the cyclinA/CDK2 complex [103], indirectly with the cyclin E/CKD2 complex through direct binding to p107 [104], and with E2F1 independent of RB [105]. It also binds the CDK inhibitors $p21^{Cip1}$ [106] and $p27^{Kip1}$ [107], and proteins important for interferon signaling such as IRF3 [108], IRF1 [109] and IRF9 (p48) [110]. Many of the E7 interactions impinge on E2F activity to enhance cell cycle progression, but likely have many other effects. Indeed E7, like large T, perturbs genome stability, and intriguingly, this activity is RB-independent [111, 112].

Adenovirus E1A comes in large (13S) and small (12S) versions due to alternative splicing. The large version has four conserved regions (CR1-4), with CR3 missing in the smaller protein. RB family members interact with the LxCxE motif in CR2, dislodging them from E2F proteins. The other regions bind numerous factors. Cr3 is a Zn finger activation domain that binds MED23 (originally known as SUR2) a member of the Mediator complex important for transcriptional regulation [113]. CR1 binds the CDK inhibitors $p21^{Cip1}$ [114, 115] and $p27^{Kip1}$ [116, 117], p400, a DNA helicase of the SWI/SNF family of chromatin remodeling complexes [118], the related HATs CBP and p300 [119, 120], the HAT PCAF, and TRAPP, a component of several HAT complexes [121]. In addition, both CR1 and CR3 bind components of the proteosome [122, 123].

In summary, these data emphasize that viral oncoproteins alter the function of many proteins in addition to Rb family protein members. Therefore, it cannot be assumed that every effect of these oncoproteins in any tissue is due to loss of Rb activity. The effects of large T/small t, E7 and E1A in the mouse retina are summarized below and in Table **2**.

Effects of SV40 T/t in the Retina

The first inheritable model of retinoblastoma utilized a transgene consisting of the SV40 early region, which expresses both large T and small t antigens, under the control of the **luteinizing hormone β-subunit gene** promoter (***LHβ-T/t***) [124]. It was designed to drive expression in the pituitary gland, but in one mouse integration on chromosome 4 resulted in expression in the retina and induction of retinoblastoma [124]. Histological and marker analyses suggested many similarities to the human disease. The cells that express T/t have not yet been described but marker analysis implied features consistent with a neuronal INL cell of origin [125, 126].

In another model, the human **phenylethanolamine N-methyltransferase** promoter was to used to direct T/t expression specifically to retinal amacrine and horizontal neurons (***PNMT-T/t***) [127, 128]. Large T was not present at P0, but was detected by P3 in horizontal cells and a subset of amacrine cells. From 3-6 weeks cells expressing T decreased in the central but not peripheral retina, and this reduction was associated with pyknosis (implying apoptosis). Only a fraction of all horizontal cells express PNMT, yet all eventually died in the central retina implicating cell non-autonomous effects [128]. The death-resistant cells in the periphery expanded at the same time as cells in the central retina disappeared and peripheral tumors were evident by 6-12 weeks [127, 128]. Immortalized cell lines from these tumors expressed amacrine and horizontal markers [127].

Large T has also been targeted to photoreceptors, using the human or mouse **interphotoreceptor retinoid-binding protein** promoter **(*IRBP-T/t*)** [129, 130] or the mouse ***rhodopsin*** **promoter (*rho-T/t*)** [131] with strikingly different outcomes. ***IRBP*** is induced at the early stages of both rod and cone photoreceptor development and continues to be expressed in mature photoreceptors [132]. Thus, the ***IRBP-T/t*** transgene is likely active in newborn photoreceptor transition cells. Tumors in ***IRBP-T/t*** transgenic mice appeared with 100% penetrance at embryonic or early postnatal stages [129, 130]. Tumors were poorly differentiated and exhibited Homer-Wright rosettes, but not Flexner-Wintersteiner rosettes, a common feature in the human disease. Genes expressed at early stages of photoreceptor differentiation, such as IRBP and S-antigen (rod arrestin) were detected, but not late-stage markers such as rhodopsin, consistent with transformation of photoreceptor transition cells [129, 130]. By ten weeks of age, large tumors were detected invading the iris and optic nerve [130]. In addition to retinoblastoma, these mice also develop pineal tumors, consistent with IRBP expression in this tissue [129, 130].

In stark contrast to ***IRBP-T/t***, a ***rho-T/t*** transgene induced photoreceptor death in the retina rather than transformation [131]. At P10, when retinal cell division is normally over, many T/t expressing cells in the ONL underwent DNA synthesis, which was associated with rapid degeneration. However, dissociated retinal cells from P10 mice grew well in attached monolayers and, unexpectedly, generated tumors when injected subcutaneously into nude mice. In addition, despite the absence of retinal tumors, ***rho-T/t*** mice developed pineal tumors [131]. Thus, the normal response to T/t-induced ectopic mitosis in late stage rods is death (***rho-T/t***), but this protective reaction requires unknown factors specific to the retinal environment. In contrast, early expression of T/t in rod transition cells (***IRBP-T/t***) results in transformation.

Effects of HPV E7 in the Retina

The ***IRBP*** **promoter** was also used to drive expression of HPV E7 in newborn photoreceptor transition cells (***IRBP-E7***) [133]. The overall histology of the E16 transgenic retina was normal, although studies were not done to assess division or apoptosis at this stage. By P1, apoptotic cells were evident in the outer retina, and by P10 cell death eliminated photoreceptors, preventing formation of the ONL. The authors then asked whether the E7-induced apoptosis was p53-dependent. In the original study, only one four week old ***IRBP-E7;p53$^{-/-}$*** retina was reported which had an emerging tumor derived from photoreceptor transition cells. At the time, the authors concluded that E7 alone induced apoptotic degeneration, and that p53 loss facilitated trasformation by blocking apoptosis. Subsequently, however, they discussed a more detailed time course analysis which revealed that the kinetics of photoreceptor apoptosis is only slightly delayed [134]. Others have since shown that p53 does not mediate photoreceptor apoptosis in the ***Rb$^{-/-}$*** retina [62]. p53 absence must facilitate photoreceptor transition cell transformation by other mechanisms possibly by decreasing genome stability and/or by altering cell motility and invasiveness [135, 136].

In another study, Griep ***et al.*** generated transgenic mice to study the effect of HPV E6 and E7 co-expression in the lens by placing the **HPV E6/E7** cassette under the control of the **α-crystallin promoter** [137]. On an FVB background these ***αAcry-HPV16E6/E7*** mice developed lens tumors. However, when one line was crossed onto a C57BL background, the F1 hybrids developed tumors, 90% of which had characteristics indicating retinoblastoma, including Homer-Wright rosettes, basal bodies and trilaminar membranes [138]. In contrast, < 1% of tumors in the FVB background were retinoblastoma. Sporadic tumors in the F1 mice arose from the INL, similar to several other models discussed here. ***In situ*** hybridization suggested that the difference in tumor susceptibility between FVB mice and B6/FVB F1 mice was not due to the expression pattern of the E6/E7 transgene. The transgene was on chromosome 12, thus had not disrupted the ***Rb*** locus on chromosome 14.

These studies prompted Griep ***et al.*** to evaluate the effect of background on the ***LHβ-T/t*** model discussed earlier. These mice were originally on a C57BL/6 background and after two generations of breeding to FVB mice tumor incidence fell by 25% [138]. Thus the FVB background protects against retinoblastoma in at least two different models.

FVB mice are homozygous for the ***rd*** allele, which causes rod apoptosis between P10 and P20. To test whether rods may facilitate INL tumor growth, the tumor prone F1 hybrids were crossed with mice carrying a transgene that expresses diphtheria toxin under the control of the ***rhodopsin*** promoter. The resultant rod death decreased tumor frequency, indicating that rod loss contributes at least part of the protective effect in FVB mice [138]. However, killing rods was not as protective as crossing mice onto the FVB background, thus FVB recessive alleles other than ***rd*** reduce the penetrance of retinoblastoma. Indeed, generating F1 hybrids with C3H mice increased retinoblastoma frequency from ~ 1% to ~40% of mice even though C3H also have the ***rd*** allele. The protective FVB alleles other than ***rd*** have not been localized, and the reason why photoreceptors promoted INL-derived retinoblastoma in this context is also still unsolved.

Effects of Adenoviral E1A in the Retina

No inheritable retinoblastoma models utilize E1A. However, as well as the early experiments with intact adenovirus (Table **1**), recent studies have utilized retroviral vectors expressing the large 13S form of E1A [139, 140]. Retroviral delivery of E1A at P0 leads to deregulated proliferation that requires the Rb binding motif [139]. E1A alone does not induce tumors but, similar to ***IRBP-E7*** mice discussed above, the absence of p53 facilitates transformation [140]. The authors suggest that the tumors may derive from RPCs, which is one possibility. Retroviral vectors require nuclear membrane breakdown at mitosis so that proviral DNA can integrate and initiate expression, thus integration occurs in RPCs. However, retroviral regulatory elements drive expression in RPCs, RTCs and terminally differentiated cells. Thus it is also possible that tumors in this model derive from RTCs or even a later stage cell type.

Do DNA Viruses Contribute to Some Cases of Human Retinoblastoma?

Virally induced models of retinoblastoma raised the possibility that DNA tumor viruses might be the causative agents in the human disease. HPV DNA was reported in retinoblastoma tumors from Mexican and South American patients. [141-144]. However, several issues remain unresolved, including whether the virus was tumor-specific or perhaps from infected conjuctiva (a known source of HPV), whether virus components were synthesized in tumors cells, or

whether viral copy number was high enough to target every tumor cell. Others reported no evidence of HPV or four other DNA viruses in human retinoblastoma [145]. Another group analyzed 39 tumors, several of which had no or only one diagnosed RB mutation, thus favoring the possibility of finding an alternative initiating event [146]. Importantly, none of five human DNA viruses were detected, although contaminating hybrid SV40-plasmid sequences common in molecular labs were detected at very low levels in some samples. Other groups have not performed such quantitative analysis, which would reveal whether there are only low levels of true virus sequences in some samples. In summary, further analyses are required to test whether HPV is a causative agent in human retinoblastoma, especially whether viral oncoproteins are present in tumor cells.

GERM LINE AND CHIMERIC KNOCKOUT MOUSE MODELS (SEE TABLES 3 & 4)

RB*$^{+/-}$** humans almost inevitably develop retinoblastoma by the age of three. This finding reflects the high probability that some ***RB*$^{-/-}$** cells will be generated during retinal development. In contrast, ***Rb*$^{+/-}$** mice do not display any retinal phenotype, but develop pituitary adenocarcinomas, thyroid medullary carcinomas and neuroendocrine tumors (Table **3**) [47, 147-150]. As in human retinoblastoma, appearance of these tumors is accompanied by the loss of the wild-type ***Rb allele [147, 150].

Mice lacking both copies of the ***Rb*** gene die around E14.5, displaying considerable proliferation and apoptosis in the CNS and PNS and defective hematopoiesis [46-48]. These early defects in the neural and erthyroid systems appear to be an indirect consequence of hypoxia due to placental defects, since ***Rb*$^{-/-}$** embryos with wild type extraembryonic tissue survive to birth [151, 152]. These mice die from respiratory problems due to defective skeletal muscle development [152]. Similar defects in myogenesis were reported previously in mice carrying a hypomorphic ***Rb*** minigene in which Rb is expressed in a subset of tissues, including the nervous system and, presumably, the placenta [52].

To overcome the lethality of germ line ***Rb*** mutations, ***Rb*$^{+/-}$** or ***Rb*$^{-/-}$** embryonic stem (ES) cells were introduced into wild type blastocysts to generate chimeric mice. Chimeric animals generated using ***Rb*$^{+/-}$** ES cells did not have an eye phenotype and the heterozygous cells contributed up to 50% of the retina. When ***Rb*$^{-/-}$** ES cells were used, the retina was normal up to E14.5, but ectopic mitosis and cell death was observed in the inner layer of the developing retina between E16.5 and E18.5 (Table **4**) [153, 154]. In this case, ***Rb*$^{-/-}$** cells contributed to only about 20% of the adult retina [153, 154]. These data suggested that Rb has an important cell

Table 3. Germline Knock-Out Mice

Genotype	Retinal phenotype/tumors	Other neuronal phenotypes/tumors Survival	References
***Rb*$^{+/-}$**	none observed	pituitary tumors	[46-48, 150]
Rb-/-	ectopic division and cell death at E13.5; e***x-vivo*** cultured retinas show ectopic proliferation and decrease in rod numbers.	ectopic proliferation/death in the lens, CNS and PNS die between E13.5-15.5	[46-48, 53, 139, 156]
TR-*Rb*$^{-/-}$ (tetraploid rescue)	none reported; presumably same as in Mox-2-Cre mice (see Table 5)	cell death in the CNS rescued; ectopic division/death in the lens not rescued die at birth	[151, 152]
***p107*$^{-/-}$**			
129/Sv: C57BL/6J BALB/cJ	none observed none observed	increased division/death in neural precursors/stem cells	[39, 40, 215, 216]
***p130*$^{-/-}$**			
129/Sv: C57BL/6J	none observed	none reported	[39]
BALB/cJ	reduced size and extensive apoptosis in the optic vesicle and stalk at E10.5	ectopic division/death in the CNS die between E11-13	[217]
***p107*$^{-/-}$*;p130*$^{+/-}$** ***p107*$^{+/-}$*;p130*$^{-/-}$**	none observed	none reported	[39, 40]
***p107*$^{-/-}$*;p130*$^{-/-}$**	none reported	none reported die at birth	[39]
***Rb*$^{+/-}$*;p107*$^{+/-}$**	none reported	pituitary tumors	[40]
***Rb*$^{+/-}$*;p107*$^{-/-}$**	dysplasia in 10% eyes in the ONL	pituitary tumors poor viability	[40]
***Rb*$^{-/-}$*;p107*$^{-/-}$** ***Rb*$^{-/-}$*;p130*$^{-/-}$**	none reported	enhanced apoptosis in the CNS die between E10.5-12.5	[40, 218]
***Rb*$^{+/-}$*;p130*$^{-/-}$**	none observed	reduced viability	[63]

CNS: central nervous system; ONL: outer nuclear layer; PNS: peripheral nervous system

Table 4. Chimeric Mice

Mutant cells	Retinal phenotype/tumors	Other neuronal phenotypes/tumors	References
***Rb*$^{+/-}$**	none observed	pituitary tumors	[153, 154]
***Rb*$^{-/-}$**	cell death	pituitary tumors	
***Rb*$^{+/-}$*;p107*$^{-/-}$**	dysplasia in 10% eyes	pituitary tumors	[60, 63]
***Rb*$^{-/-}$*;p107*$^{-/-}$**	**retinoblastoma** 5/7 mice, >1 mo, INL, amacrine cell markers, HW rosettes	none reported (poor viability)	[60]
***Rb*$^{+/-}$*;p130*$^{-/-}$**	none reported	none reported	[63]
***Rb*$^{-/-}$*;p130*$^{-/-}$**	**retinoblastoma** 5/11 mice, ~3 mo, INL, amacrine cell markers, HW rosettes	none reported	[63]

INL: inner nuclear layer; HW: Homer-Wright

autonomous role in cell cycle exit and survival in most retinal cells. However, whether all or only specific cell types required Rb for these functions was not clear. As we discuss below, this question was answered using CKO models.

Retinoblastoma was still not observed in chimeric mice generated with ***Rb*$^{-/-}$** cells. Instead, the animals succumbed to pituitary tumors between the ages of 3 and 11 months [153, 154]. Apart from adrenal neoplasia, no other tumors were observed. Thus, loss of Rb alone in the mouse retina results in ectopic proliferation and cell death but not transformation, suggesting a requirement for additional mutations. Subsequently, a major breakthrough came when chimeric mice were generated using ***Rb*$^{+/-}$*;p107*$^{-/-}$** or ***Rb*$^{-/-}$*;p107*$^{-/-}$** ES cells [60]. ***Rb*$^{+/-}$*;p107*$^{-/-}$** ES cells generated chimeras at normal rates, but a fraction (6/56) of eyes exhibited retinal dysplasia, which had also been noted in some ***Rb*$^{+/-}$*;p107*$^{-/-}$** mice (Table **4**) [40]. When ***Rb*$^{-/-}$*;p107*$^{-/-}$** ES cells were used, chimeras were generated only when low numbers (4 to 6 cells) were used per injection [60]. Even then, few live births were observed (7/56) and the contribution of ES cells in adults was about two-fold lower than when ***Rb*$^{-/-}$** cells were used. Most importantly, however, retinoblastoma was observed in 6/14 eyes, proving that p107 is a tumor suppressor in the Rb-deficient mouse retina [60]. Recently, chimeras were generated using ***Rb*$^{-/-}$*;p130*$^{-/-}$** ES cells and these mice also developed retinoblastoma (Table **4**) [63]. These animals were easier to generate than ***Rb*$^{-/-}$*;p107*$^{-/-}$** chimeras, suggesting a more important role for p107 than p130 in compensating for the absence of Rb during development. In summary, mice, and likely other animals, resist retinoblastoma due to the protective effect of Rb relatives.

These studies also provided some initial insight into the potential cell of origin of retinoblastoma. Tumors in ***Rb/p107*** or ***Rb/p130*** DKO chimeras arose from the INL, had nuclear staining patterns like INL cells, and many cells expressed markers of amacrine neurons, such as syntaxin, and GABA [62, 63]. Some tumors also contained activated Müller glia, either implicating a cell of origin capable of generating both amacrine and Müller glia, but more likely representing gliosis in response to retinal damage. As noted above, retinoblastoma in several transgenic models also has an INL origin and, intriguingly, small retinoblastoma tumors have been seen emerging from the INL in humans [59].

CONDITIONAL KNOCKOUTMOUSEMODELS (SEE TABLE 5)

Chimeric models of retinoblastoma are technically difficult to generate, provide limited numbers of animals due to high rates of lethality, are not inheritable, and generate variable proportions of knockout cells in the retina which are difficult to identify without appropriate marker transgenes in the donor ES cells. CKO approaches using the Cre-loxP system have been used to overcome these drawbacks (Table **5**). Here, targeted expression of Cre recombinase in the developing retina excises ***Rb*** exons flanked by loxP sites ("floxed" ***Rb*** or ***Rb*$^{f/f}$**). The two most comprehensive CKO studies analyzed both tumorigenesis and the cell-type specific effects of deleting one or more ***Rb*** family members [61, 62]. Another study generated a third CKO model of retinoblastoma [140]. Below, we outline the approaches used then, in a series of sections, discuss the implications of these studies with regards to the timing of Rb action, the cell of origin of retinoblastoma, the cell specific responses to Rb loss, the function of post-***Rb*** mutations in retinoblastoma, how the cell of origin might explain the existence of pediatric tumors, the role (or not) of p53 in retinoblastoma, and why p107 or p130 prevent retinoblastoma in the mouse but not human ***Rb*$^{-/-}$** retina.

α*-Cre Mice*

The α***-Cre*** transgene utilizes the ***Pax6*** α-enhancer to drive Cre expression beginning at around E10 in most of the peripheral retina. This expression pattern ensures that floxed alleles are deleted prior to differentiation so that all RPC, transition and mature cells lack the target gene. RPCs in the central and dorsal peripheral retina do not express Cre so these regions act as a useful

control. The α***-Cre*** gene gradually turns off again during embryonic retinal development, but is expressed in a subset of mature amacrine and ganglion cells throughout the whole retina. Chen ***et al.*** used these mice to study the effect of Rb loss on retinal development either in the presence or absence of p107 [61]. 60% of α***-Cre;Rb$^{f/f}$;p107$^{-/-}$*** eyes develop retinoblastoma which are visible by P8. Mature tumors express markers of amacrine cells such a syntaxin and calretinin, and emerging small tumors contain cells expressing the combinatorial code of transcription factors that determinants the amacrine cell lineage (NeuroD and Math3) [61]. Notably, young and old tumors lack Chx10, a marker of RPC and bipolar cells, and they also lack other mature neuronal markers. Some cells express Müller markers, consistent with a gliotic response. These data suggest that tumors arise from amacrine transition cells.

Nes-Cre1 Mice

The ***Nes-Cre1*** transgene exploits a Nestin promoter to drive Cre expression in multiple neuronal and other tissues. In ***Nes-Cre1*** mice, expression is more extensive when the transgene is inherited paternally (***Nes-Cre1(p)***), presumably due to imprinting effects. Gene inactivation occurs in the optic vesicle at E9.5 generating a completely Rb-deficient retina [62]. Wider expression in the CNS and other tissues also results in neonatal lethality of ***Nes-Cre1(p);Rb$^{f/f}$*** mice. Maternal inheritance (***Nes-Cre1(m)***) results in mosaic and highly variable Cre expression in each offspring. No adult ***Nes-Cre1(m);Rb$^{f/f}$;p107$^{-/-}$*** mice were obtained, presumably due to lethality prior to weaning. However, ***Nes-Cre1(m); Rb$^{f/f}$;p130$^{-/-}$*** animals were obtained at ~half the expected Mendelian frequency and of five animals obtained, 10/11 eyes had retinoblastoma [62]. As in the α***-Cre*** model, tumors expressed markers of the amacrine cell lineage.

Chx10-Cre Mice

In this CKO model, the ***Cre*** transgene consisted of a bacterial artificial chromosome (BAC) vector in which expression was under the control of ***Chx10*** regulatory elements [140]. Normally, Chx10 is expressed in all cells of the early retina, but the ***Chx10*** BAC vector, like the ***Nes-Cre1(m)*** transgene, provides mosaic expression. Tumors were observed in ***Chx10-Cre;Rb$^{f/f}$;p107$^{-/-}$*** mice. An attractive feature of mosaic models, such as the ***Nes-Cre1(m)*** and ***Chx10-Cre*** mice is that they recapitulate sporadic inactivation of ***Rb***, similar to spontaneous ***RB*** loss in the developing human retina. A practical

Table 5. Conditional Rb Knock-Out Mice

Cre Promoter/ Spatial activity	Time /Area of retinal KO	Retinal phenotype and tumors in Rb^{ff} (floxed) mice	References
α**-Pax6** retina	E10.5 peripheral retina	Division/survival of RPCs not affected. Ectopic division of all RTCs. Ganglion, bipolar and half of rod photoreceptor cells die. ***p107$^{-/-}$*** **bckd:** as RbKO except only amacrine, horizontal and Müller cells survive. Tumors seen as early as P8 in the INL, displaying amacrine cells markers and HW rosettes.	[61, 62]
Nestin CNS,lens	E9.5		[62]
paternally inherited	whole retina	Ectopic division and death at E18.5. Aberrant GCL morphology. (Mice die at birth.) ***p53$^{-/-}$*** **bckd:** Cell death not rescued. Focal dysplasia.	
maternally inherited	mosaic	Cell death in the ONL. Mature ONL reduced by half, outer segments diminished. Disorganized INL. ***p53$^{-/-}$*** **bckd:** Cell death not rescued. Focal dysplasia. ***p107$^{-/-}$*** **bckd:** Massive retinal dysplasia, apoptosis at E18.5. Die prior to weaning. ***p130$^{-/-}$*** **bckd:** No dysplasia/apoptois at E18.5 but tumors develop in the INL of adults displaying HW rosettes and amacrine cell markers.	
Chx10-BAC retina	E9.5 mosaic RPCs, bipolar cells,a subset of Müller glia	Number of rods reduced, disruption of the OPL. ***p107$^{+/-}$*** **bckd:** Dysplasia. Ectopic division/death/ reactive gliosis. Ectopically dividing cells expressing markers incl. those of Müller and amacrine cells. ***p107$^{-/-}$*** **bckd:** tumors expressing cell markers incl. those of amacrine cells. ***p53^{ff} ; p107$^{-/-}$*** **bckd:** tumors displaying rosettes and expressing markers incl. those of amacrine cells. ***p53^{ff};p107$^{+/-}$***, ***p53$^{+/-}$;p107$^{-/-}$*** and ***Rb$^{f/+}$; p53^{ff} ;p107$^{-/-}$*** **bckd:** also display tumors.	[139, 140, 159, 219, 220]
IRBP retina pineal gland	E13 rod/cone RTCs	No phenotype in the ONL observed even on ***p107$^{-/-}$***, ***p53$^{-/-}$*** or ***p107$^{-/-}$;p53$^{-/-}$*** **bckd.**	[164]
Mox2 embryo proper (not placenta)	E6.5 whole retina	None reported ***in vivo***. (Mice die at birth). E18.5 retinas cultured ***ex-vivo*** for 12d show reduction of rods.	[139, 151, 152]

Bckd: background; CNS: central nervous system; GCL: ganglion cell layer; HW: Homer-Wright; INL: inner nuclear layer; ONL: outer nuclear layer; RPC: retinal progenitor cell; RTC: retinal transition cell

disadvantage is that is not straightforward to define clones of KO cells. The peripheral KO region in the α-Cre model can be spotted easily, although the edge of the KO region is not sharp, generating a region around the border that is mosaic.

These three different approaches to conditionally inactivate ***Rb*** all generated novel models of inheritable retinoblastoma that confirm the chimeric studies showing that, in the mouse, retinoblastoma requires loss of both ***Rb*** and at least one of its relatives, ***p107*** or ***p130***. Below, we outline novel information and ideas that these models have spawned.

INSIGHTS FROM CONDITIONAL KNOCKOUT MOUSE MODELS

1. Rb Blocks Division in Newborn Transition Cells

Overview: ***Rb loss triggers ectopic division of all differentiating RTCs. There is no clear-cut evidence that Rb loss affects RPC expansion, and indeed there is evidence against it. These findings implicate the ectopically dividing RTC as the starting point for retinoblastoma.***

To define the cell of origin of retinoblastoma, it is important to determine the critical function(s) of Rb in the developing retina, particularly to decipher when and where the protein acts first. In view of its role in cell cycle inhibition, Rb could be important to block division in newborn RTCs and/or might be required to temper the expansion of RPCs. BrdU labeling of ***Rb*** knockout (***Rb***KO) retinal sections revealed abnormal division [61, 62]. Many ectopically dividing cells could be seen in the inner retina, including the GCL, where there are no RPCs (Fig. **1C**) [61, 62]. p107 loss alone had no effect, but exasperated the frequency of ectopically dividing cells in combination with Rb loss [61].

There was an outside chance that these cells were ectopically placed RPCs, but their abundance argued in support of the simpler interpretation that they are abnormally mitotic RTCs. More emphatically, the markers Brn3b and Crx, which are expressed in ganglion cells and rod/cone photoreceptors, respectively, never co-localize with BrdU in the WT retina [155] (Furukawa, personal comm., D. Chen and R.B., unpublished), but many Brn3b cells in the inner RbKO or Rb/p107 DKO retina were $BrdU^+$ and many Crx^+ cells on the outer third of the retina were $BrdU^+$ (Fig. **1C**) [61]. Other markers revealed ectopically dividing amacrine, horizontal, Muller and bipolar cells [61].

We confirmed these results in a sporadic model of ***Rb*** deletion that exploits Cre-GFP retrovirus delivery at P0. Quadruple labeling was used to mark cells transduced with virus (GFP^+), the specific RTC marker, BrdU incorporation, and DAPI to mark nuclei and define retinal layering. In this case, 8 markers were used, two each for the four cell types born in the post-natal retina. In all 8 cases we detected ectopic division in both Rb and Rb/p107 deficient clones (Fig. **1C**, M.P. & R.B., unpublished results). We confirmed that these markers are not present in $BrdU^+$ RPCs of the WT retina, so they exclusively tag differentiating RTCs. These data show that Rb is not necessary for the specification of RTCs or migration to their final destination, but couples RTC birth to cell-cycle exit (Fig. **1D**). Alone, p107 loss has no effect, but exacerbates ectopic division in the absence of Rb.

We investigated the role of different E2Fs in driving ectopic RTC division (D. Chen, M.P. & R.B. unpublished data). In the α-Cre model, absence of E2F1 completely rescues all ectopic division in the absence of Rb whereas loss of E2F2 or E2F3 has no effect. Similarly, E2F1 deletion suppresses ectopic division in sporadic ***Rb***KO or ***Rb/p107*** DKO clones generated with Cre-GFP virus. Thus, E2F1 drives ectopic RTC division in the absence of Rb (Fig. **1D**). This finding is distinct from other ***Rb***KO CNS regions where either E2F1 or E2F3 deletion suppresses ectopic division [53, 156, 157].

The above function for Rb is consistent with its role in other contexts. For example, as we discussed earlier, in the WT forebrain differentiating cells migrating through the neuroepithelial sheet are β-III $tubulin^+$ and post-mitotic. In the absence of Rb these cells incorporate BrdU [51]. Similarly, Rb is crucial to block division in differentiating muscle and lens fibroblasts [49, 52]. Thus, Rb drives cell cycle exit in differentiating cells in numerous contexts, and specifically in RTCs in the retina.

In addition to this function, does Rb also play a role in tempering RPC cell expansion in the retina? If this were the case, then there should be extra RPCs in the ***Rb***KO retina. Two results from the ***α-Cre*** model argue against this possibility. First, at E17 Chx10 marks only RPCs and the number of $Chx10^+/BrdU^+$ cells was not altered in the ***Rb/p107*** DKO retina [61]. Second, RPCs undergo mitosis on the outermost surface of the neuroretinal sheet (Fig. **1A**) and the number of mitotic RPCs adjacent to the outer limiting membrane was not altered at E17 or P0 either in the absence of Rb or both Rb and p107 [61]. The same result was observed in the E18 RbKO retina using the ***Nes-Cre1*** model [62]. Thus Rb or Rb/p107 loss does not appear to have any detectable effect on RPC division.

These data fit the expression of Rb and its negative regulators. In the embryonic mouse, very few, if any, RPCs express Rb mRNA or protein [158, 159]. Embryonic time points are critical since tumor cells in KO models have the characteristics of amacrine cells, most of which are born around E15 (Fig. **1B**). Second, although more RPCs express Rb in the post-natal retina [139, 159], cyclin D1 levels are much higher in the developing retina than any other tissue and likely maintain Rb in an inactivate state until RTC birth occurs [160]. Indeed, cyclin D1 deletion results in a reduction in retinal cell number [160]. This drop can be seen as early as E16.5 which, given the virtual absence of Rb in RPCs at

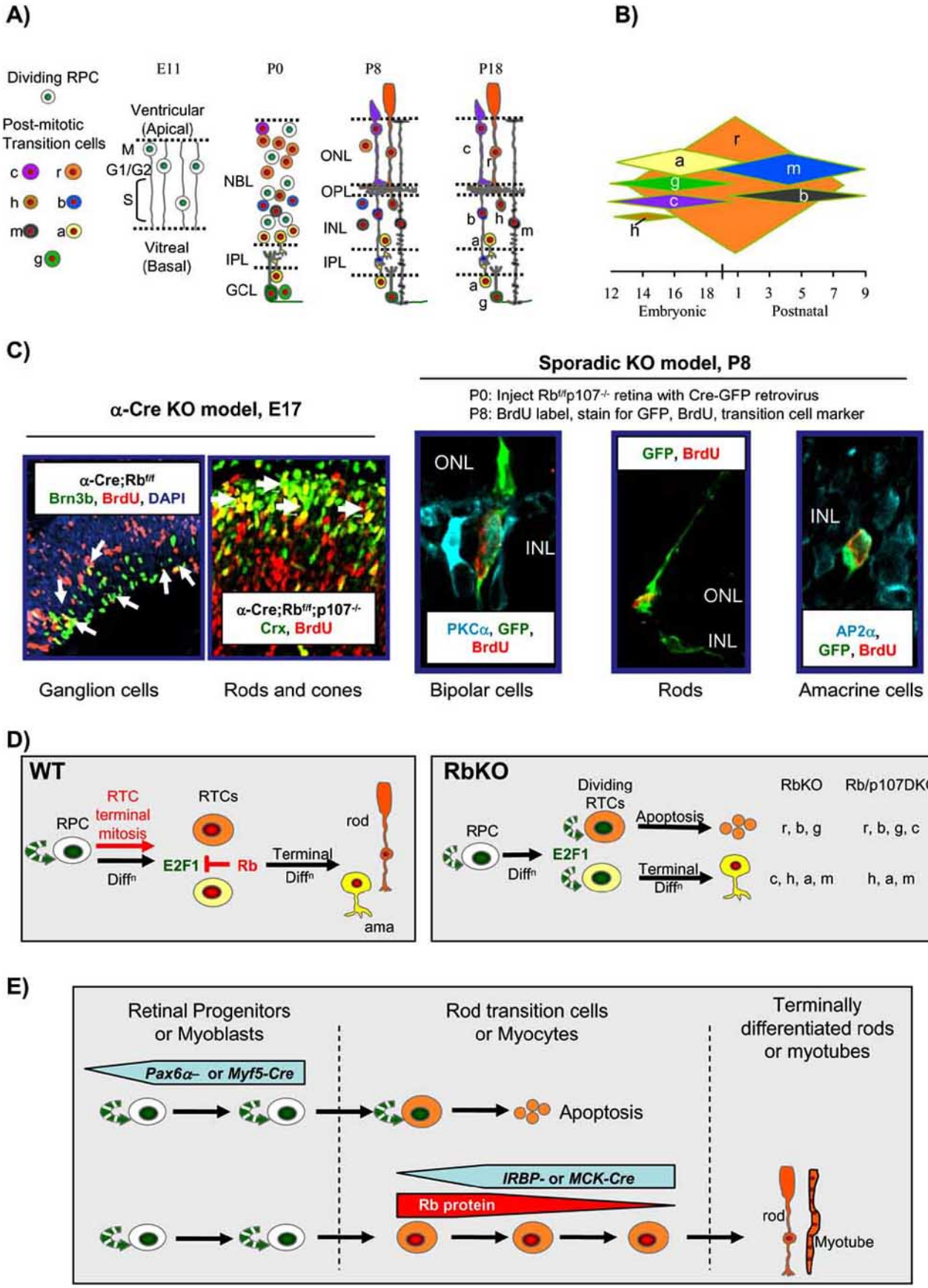
A)
Dividing RPC
Post-mitotic Transition cells
c r h b m a g
E11
Ventricular (Apical)
M
G1/G2
S
Vitreal (Basal)
P0
NBL
IPL
GCL
P8
ONL
OPL
INL
IPL
P18
c r b h m a a g
B)
r a m g b c h
12 14 16 18 1 3 5 7 9
Embryonic
Postnatal
C)
α-Cre KO model, E17
Sporadic KO model, P8
P0: Inject Rb^f/f p107^-/- retina with Cre-GFP retrovirus
P8: BrdU label, stain for GFP, BrdU, transition cell marker
α-Cre;Rb^f/f
Brn3b, BrdU, DAPI
α-Cre;Rb^f/f;p107^-/-
Crx, BrdU
ONL
INL
PKCα, GFP, BrdU
GFP, BrdU
ONL
INL
INL
AP2α, GFP, BrdU
Ganglion cells
Rods and cones
Bipolar cells
Rods
Amacrine cells
D)
WT
RPC
RTC terminal mitosis
Diffn
RTCs
E2F1
Rb
Terminal Diffn
rod
ama
RbKO
RPC
Diffn
Dividing RTCs
E2F1
Apoptosis
Terminal Diffn
RbKO
Rb/p107DKO
r, b, g
r, b, g, c
c, h, a, m
h, a, m
E)
Retinal Progenitors or Myoblasts
Rod transition cells or Myocytes
Terminally differentiated rods or myotubes
Pax6α– or Myf5-Cre
Apoptosis
IRBP- or MCK-Cre
Rb protein
rod
Myotube

Fig. (1). contd.....

Fig. (1). Rb controls cell cycle exit in newborn transition cells. **A)** Retinal Development. At E11 the retina consists of a neuroblastic layer (NBL) made up of dividing RPCs (white circle, green nuclei). RPC cell bodies oscillate in relation to cell cycle phase. At P0 the NBL contains a mix of RPCs and post mitotic RTCs (coloured circles, red nuclei) and is separated from the differentiated ganglion cell layer (GCL) by the inner plexiform layer (IPL). By P8 there are no RPCs, fewer RTCs and more differentiated rods (r), cones (c) horizontal (h), bipolar (b) Müller (m) amacrine (a) and ganglion (g) cells. Retinal maturation is complete by P21. **B)** The time period when each RTC is born (exits the cell cycle) is shown. **C)** Ectopic division of Rb- or Rb/p107-deficient RTCs. Two panels on the left show sections from E17 RbKO or Rb/p107 DKO retina stained for dividing cells (BrdU, red) and differentiating ganglion cells (Brn3b, green). Three panels on the right are from P8 ***Rb$^{f/f}$*** retina (***^{f}***: floxed allele) infected ***in vivo*** with Cre-GFP retrovirus at P0. All sections were stained for virus-transduced (GFP, green) and dividing (BrdU, red) cells. The outer panels also show markers for bipolar (PKCα, blue) and amacrine (AP2α, blue) cells. The GFP$^+$/BrdU$^+$ cell in the center panel is a rod transition cell (located in ONL, and expresses rod arrestin, not shown). Ectopically dividing Müller glia are also common in this model (not shown). The cell type markers used are only found in post-mitotic cells in the wild type retina (M.P. and R.B., unpublished). Thus, Rb is critical to block division in RTCs. **D)** Model of Rb action. In the WT retina, RPCs divide (green nuclei, striped arrow) and generate post-mitotic RTCs (red nuclei). Rb blocks division in RTCs by inhibiting E2F1. In the ***Rb***KO retina, RTCs are born as usual but divide due to unleashed E2F1 activity. The green and white striped arrow indicates Rb-deficient RTCs have a finite proliferative capacity. Some ectopically dividing cells die while others survive and exit division independent of Rb/p107 as they terminally differentiate (cell type abbreviations as in A). **E)** Temporary exposure of RTCs or myocytes to Rb protein is sufficient to ensure permanent cell cycle exit and normal terminal differentiation. In α***-Cre*** or ***MYF5-Cre*** mice, the ***Rb*** gene is deleted early in retinal and muscle development, so Rb protein is completely absent as RTCs or myocytes are generated. Rb absence triggers ectopic division (green nuclei and striped arrow) which causes apoptosis of several types of RTCs (e.g. rods) and also of myocytes. In contrast, in ***IRBP-Cre*** or ***MCK-Cre*** mice, time is required for the accumulation of Cre protein, and the decay of Rb mRNA and protein. The short exposure to Rb is sufficient to ensure permanent cell cycle exit (red nuclei). Moreover, terminal differentiation occurs even if Rb is absent.

that time, may be due either to the associated increase in the CDK inhibitor p27^{Kip1} in RPCs [161] and/or activation of p107, which is present in embryonic retinal RPCs [158, 159].

Dyer's group has suggested that Rb does temper RPC expansion in the retina [139, 159]. They deleted Rb using either a ***Chx10-Cre*** transgene or a retroviral Cre vector and saw extra BrdU labeled cells and concluded they must be RPCs [139]. However, these studies were done on dissociated cells, so valuable positional information was lost, such as the abnormal division of ganglion and amacrine cells in the inner retina which was apparent when retinal sections were analyzed in α***-Cre*** or ***Nes-Cre*** models [61, 62]. Moreover, double labeling was not performed, so dividing Brn3b$^+$/BrdU$^+$ ganglion cells or Crx$^+$/BrdU$^+$ rods and cones were missed.

Subsequently, the same group used two different approaches to investigate whether Rb loss generates extra RPCs. First, they reported that Rb loss triggers the production of more cells that express "RPC" markers [159]. However, it has never been proven that these markers are unique to RPCs, and the authors who first identified them cautioned that they may, like many RPC-enriched genes, be expressed in some newborn RTCs either for a short or extended period after cell birth [162]. Moreover, it is likely that genes normally restricted to RPCs in the WT retina continue to be expressed in ectopically dividing RTCs. This outcome is inevitable for molecules that play a role in driving the cell cycle (some of which were used to mark "RPCs" by Dyer's group), which may, in turn, drive the expression of other RPC markers as part of a program of gene expression in cycling cells. Thus, the only reliable way to convincingly distinguish a RPC from an ***ectopically dividing*** RTC is to use markers specific to the latter and not the former.

In a second approach two markers were used to label dividing cells (BrdU and ^{3}H-thymidine), one delivered at the same time as ***Rb*** was inactivated (using a ***Cre*** retrovirus), the second delivered several days later. They reasoned that because RPCs were marked at the start of the assay with, say BrdU, then double labeled BrdU/^{3}H-thy labeled cells at the end of the experiment must be RPCs. In RbKO cells they found excess BrdU/^{3}H-Thy cells and concluded that Rb must temper RPC expansion. However, during the course of the experiment, many ***Cre***-virus-transduced RPCs would differentiate into newborn RTCs and, in the absence of Rb, continue to divide. This scenario also produces extra BrdU/^{3}H-Thy cells because ectopically dividing RTCs derived from BrdU$^+$ RPCs incorporate the second label. Thus, while double labeling with BrdU and ^{3}H-Thy at the beginning and end of an experiment can differentiate RPCs and RTCs in the WT retina, it is not a viable approach when RTCs divide ectopically, as is the case in the absence of Rb.

The debate about where Rb acts, in particular where it has its first role in retinal development, affects our interpretation of which cell type gives rise to retinoblastoma. There are as yet no clear-cut data in favor of the idea that Rb loss triggers the production of extra RPCs, but instead there is evidence to the contrary [61, 62]. Moreover, it is clear that Rb loss triggers ectopic division of differentiating RTCs (Fig. **1C**) [61, 62] (M.P. & R.B. unpublished data), a finding that is in agreement with the function of Rb in other neuronal tissues [51] and with the expression pattern of Rb and its negative regulators in the retina [158-160]. Because

the RTC is where Rb loss first triggers a proliferative defect it is an excellent candidate for the starting point of retinoblastoma. Moreover, targeting of viral oncoproteins to different RTCs proves that these cells are susceptible to transformation [127-130, 133]. Finally, deleting ***Rb*** and ***p130*** causes mouse retinoblastoma [62, 63], and since p130 is present only in RTCs not RPCs [159], and neither its expression nor phosphorylation state are affected by Rb or Rb/p107 loss [159, 163], the tumors must arise from ectopically dividing RTCs, not RPCs.

2. Rb is Required in a Limited Time Window Soon After Transition Cell Birth

Overview: ***Rb-deficient RTCs divide ectopically, but if Rb protein disappears several hours after RTCs are born, there is no effect. Thus Rb is like a key that locks the door on cell division; it can be discarded after the job is done and the door remains locked.***

In addition to the transgenic models discussed earlier, one other attempt has been made to use the Cre/loxP system to generate inheritable retinoblastoma [164]. Although no retinal tumors developed, the data provide useful insight into the timing of Rb action in RTCs. The model utilized an ***IRBP-Cre*** transgene to generate Rb-deficient photoreceptors. Two ***IRBP-Cre*** lines were investigated, one which showed Cre immunoreactivity in the outer retina at E14.5 (i.e. emerging cones and some rods) and another at P0; the former was used to knock out ***Rb***. Cre expression was not uniform, but was detected in roughly 20-40% of photoreceptors. Ectopic division and apoptosis were assessed at P7 but none was observed, and the mature ONL appeared morphologically normal. Even ***IRBP-Cre;$^{Rbf/f}$;p107$^{-/-}$*** mice had no abnormalities in the photoreceptor layer [164].

These data are in stark contrast to those from ***α-Cre*** or ***Nes-Cre1*** models, where differentiating photoreceptor transition cells divide, and half of RbKO rods and all Rb/p107 DKO rods and cones are deleted by apoptosis, with death rates peaking at around P8 [61, 62]. In ***α-Cre*** or ***Nes-Cre1*** models ***Rb*** is deleted early in retinal development [61, 62] so RTCs are born devoid of Rb (Fig. **1E**). However, in the ***IRBP-Cre*** model, ***Cre*** expression is activated as photoreceptors are born, coinciding with the expression and/or activation of Rb protein (Fig. **1E**). It would take several hours after cell birth for Cre mRNA then protein to amass, and then Rb mRNA and protein to decay. In ***IRBP-E7*** or ***IRBP-T*** transgenics [129, 133] rapid viral oncoprotein accumulation would direct much faster inactivation of Rb family proteins than Cre mediated excision of the ***Rb*** gene. Putting all these studies together, we can surmise that a relatively brief exposure to Rb is both sufficient and essential to ensure newborn photoreceptor transition cells leave the cell cycle permanently. This appears to be true in other types of neurons; while E1A expression in immature neurons triggers ectopic division, it has no effect in mature cortical neurons [165].

The timing of Rb action in RTCs has a striking parallel in differentiating skeletal muscle. When ***Rb*** is missing in the germ line, or when a ***Myf5-Cre*** transgene is used to delete ***Rb*** in myoblasts, differentiating myocytes divide and undergo apoptosis, causing death of the animals at birth [52, 166]. In contrast, when the ***MCK-Cre*** transgene is used to delete ***Rb*** in differentiating myocytes, muscle differentiation is unaffected [166]. Again, there would be several hours between Cre expression, action, and decay of Rb mRNA and protein. Thus, in either muscle or retina, Rb appears to trigger permanent cell cycle exit within a relatively narrow window of time after differentiation begins, beyond which it is no longer required.

If Rb is the key that locks the door on cell cycle progression, and can be discarded after it completes its role, what are the components of the lock that keep E2F-regulated cell cycle genes silent in the absence of Rb? Other pocket proteins may ensure permanent shut down of E2F targets. Indeed, p130 is expressed in mature retinal cells [158, 159]. In another system, early appearing RB-E2F complexes seen in differentiating HL60 cells are eventually replaced by E2F-p130 complexes [54]. In addition, while bound to E2F, Rb can recruit chromatin and/or DNA modifying enzymes that can permanently silence targeted genes. For example Rb can recruit the histone methyltrasferase (HMT) Suv39H to E2F targets [167-169]. When dividing myoblasts were induced to differentiate into post-mitotic myocytes, the level of lysine 9 methylation on histone h3 (H3meK9) was elevated [170]. In differentiating myocytes cell cycle genes are silenced and non serum responsive, but Suv39H knockdown re-sensitized these genes to serum [170]. In addition to histone methylation, Rb could also facilitate permanent shut down of cell cycle genes by recruiting DNA methyl transferases [171].

Many Rb corepressors, like viral oncoproteins, use an LxCxE motif to bind the Rb pocket domain. Knockin mice have been generated in which the LxCxE binding domain (LBD) in the Rb pocket was mutated [172]. This protein binds and quenches E2F activity, but does not recruit many LxCxE corepressors. It would be interesting to determine whether this version of Rb ($Rb^{\Delta LBD}$) can drive permanent cell cycle exit in differentiating cells or whether the resulting terminally differentiated cells are susceptible to cell cycle entry.

3. Cell Type Specific Apoptotic Responses to Rb Loss

Overview: ***Before the advent of CKO models it seemed ectopic division of RbKO cells in the developing retina produced widespread apoptosis. In fact, only some cell types are death prone. Even in the Rb/p107 DKO retina, amacrine, horizontal and Müller cells are surprisingly death-resistant.***

Studies on null mice suggested that the common response to Rb loss was ectopic division coupled to cell death [46-48]. This notion was supported by data showing that, in addition to ectopic division, IRBP driven expression of HPV E7, E2F1 or cyclin D1 drives apoptosis of photoreceptors [133, 173, 174], and E7 also triggers death in lens fibers [175]. Moreover, the extended survival of null mice carrying an ***Rb*** transgene expressed in limited subset of tissues ends near term due to apoptosis of skeletal muscle [52].

The initial notion that ***Rb*** loss always leads to apoptosis was modified by several observations. First, chimeric animals revealed that ***Rb*$^{-/-}$** cells can contribute to almost every tissue as effectively as wild type cells [153, 154]. One notable exception was the retina where much fewer KO cells survived compared to other tissues, fueling the idea that all ectopically dividing retinal cells may be death prone in the absence of Rb. Second, whereas ***Rb*** null mice showed extensive apoptosis in the CNS, conditional ***Rb*** deletion in this tissue triggered ectopic division without apoptosis [51, 163]. Finally, a recent study found that, in addition to CNS and hematopoietic phenotypes noted before, ***Rb*** null mice also exhibit severe defects in the labyrinth layer of the placenta including trophoblast cell hyperplasia and abnormal labyrinth structure that impairs placental transport function [151]. When ***Rb*** was deleted specifically in embryonic but not extra-embryonic tissue, normal placental development was associated with almost complete suppression of CNS apoptosis and hematopoeitic abnormalities, whereas ectopic division was not suppressed [151, 152]. ***Rb*** null embryos with a normal placenta survived to term but exhibited the same defects in lens and muscle as reported previously [151, 152]. Thus, ***Rb*** loss triggers cell autonomous ectopic division in multiple tissues, and cell autonomous apoptosis in muscle, lens, and retina, but in most of the CNS or in hematopoietic cells, death is an indirect effect of abnormal placental function and probable hypoxia.

These studies did not reveal whether all retinal cell types are death prone in the absence of Rb. ***Rb*** null embryos with a normal placenta die at birth and limited histological analysis of the retina at this stage did not detect gross defects [152], but ectopic division, apoptosis or effects on specific cell types were not assessed. Analysis of CKO models revealed apoptosis in embryonic and post-natal ***Rb***KO or ***Rb/p107*** DKO retina, peaking at around P8, but unexpectedly, death was cell-type specific [61]. ***Rb*** loss triggered ectopic division of all differentiating RTCs, but only ganglion and bipolar cells and about half of rod photoreceptors underwent apoptosis (Fig. **1D**) [61, 62]. In the absence of both ***Rb*** and ***p107*** cones were also deleted but, unexpectedly, amacrine, horizontal and Müller cells survived (Fig. **1D**) [61]. Loss of ***Rb/p107*** DKO photoreceptors is consistent with the observations that over-expression of E7, E2F1 or cyclin D1 all induce apoptosis in this cell type [133, 173, 174].

The molecular basis for the differential susceptibility of distinct RTCs to apoptosis in the absence of Rb family proteins is unknown. Like ectopic RTC division, apoptosis is rescued by deleting E2F1 (D. Chen, M.P. & R.B. unpublished data). Identifying the critical molecules that mediate the distinct response to E2F1 in RTCs may pave the way for novel treatments to prevent and/or treat retinoblastoma. Irrespective, these differences have important implications for the type and number of events required for tumor progression, which we discuss below.

4. Cell of Origin Influences the Nature and Number of Genetic Lesions Required for Transformation

Overview: ***Sporadic mouse retinoblastoma in Rb/p107 or Rb/p130 DKO models arises from death-resistant amacrine transition cells. Most amacrine cells escape tumorigenesis not by dieing but by exiting the cell cycle in an Rb/p107-independent fashion. Post-RB mutations are required to overcome this growth-arrest barrier. Death-prone RTCs would require at least two post-Rb mutations, one to overcome apoptosis and another to overcome growth arrest.***

The two-hits that target the ***RB*** gene are rate limiting for retinoblastoma, but do not exclude the possibility that additional non-rate limiting events are necessary for transformation. Indeed numerous extra lesions are seen in tumors, several of which are present at high frequencies (see section: “Human retinoblastoma – more than just two hits”). Tumors in conditional mouse models are sporadic, indicating that additional post-***Rb*** mutations are also critical in this context.

As discussed in the prior section, it was initially thought that all ***Rb***-deficient retinal cell types are apoptosis-prone, suggesting that post-***Rb*** mutations might overcome RTC apoptosis [59]. A unifying feature of multiple chimeric and conditional KO models in which ***Rb*** and ***p107*** or ***p130*** are inactivated is that the retinoblastoma tumors have amacrine cell characteristics [60-63]. Critically, these cells are one of the three types that are naturally death resistant in the Rb/p107 DKO retina [61]. If the cell of origin already comes equipped with an intrinsically high resistance to apoptosis (at least at the early stages), what is the purpose of post-***Rb*** mutations?

Although considerable ectopic division is evident in the late embryonic and early post-natal ***Rb/p107*** DKO retina, it diminishes and is over by ~P30 [61]. Despite ectopic division, and despite the absence of their bipolar and ganglion cell synaptic partners, amacrine cells terminally differentiate and form an inner plexiform layer [61]. Thus, in addition to Rb/p107 loss, amacrine cell transformation requires a

mutation to overcome cell cycle exit linked to terminal differentiation (Fig. **2**).

The above model does not exclude the possibility that some tumors arise from death-prone cell types. In that case, at least two post-***RB*** mutations would be required, one to inhibit apoptosis and one to over-ride eventual growth arrest (Fig. **2**). It is intriguing that later diagnosed tumors have more chromosomal alterations than those from younger children [8, 9, 12]. Such a dual event would be required to transform mouse Rb/p107 DKO photoreceptors, which are highly sensitive to apoptosis [61]. Indeed, while mouse photoreceptors do not form tumors in the absence of Rb and/or p107, they can be transformed by viral oncoproteins, which target both the Rb and other pathways (see above) [129, 130, 133]. Human retinoblastoma tumors often, but not always, express photoreceptor markers [176]. Several models may explain the greater susceptibility of ***Rb***KO human versus ***Rb/p107*** DKO mouse photoreceptor transition cells to transformation [177]. First, human rod and cone transition cells may be more death resistant than their mouse equivalents. Second, the sporadic nature of homozygous ***RB*** loss in humans generates mutant RTCs surrounded by normal neighbors. In this circumstance, mutant cells may survive longer, providing a lengthened period for the appearance of sequential mutations that inhibit apoptosis and growth arrest. Indeed, in a sporadic model of ***Rb*** deletion utilizing a Cre virus, we have observed that ectopically dividing ***Rb*** or ***Rb/p107*** DKO rods are much more death-resistant than those in models where ***Rb*** is inactivated in a large area of the retina (M.P. & R.B., unpublished). Third, if the genome in dividing human photoreceptor transition cells is unstable it would accelerate appearance of post-***RB*** mutations. Fourth, the photoreceptor markers present in human retinoblastoma samples may reflect transdifferentiation of highly malignant advanced tumors.

Earlier, we discussed the common gains in retinoblastoma at 6p22 and 1q32-34 as well as loss at 16q22 that are linked to increased levels of E2F3 and KIF14A or loss of CDH-11, respectively [14-17]. Notably, E2F3 is important in the progression from G1 to S phase [157, 178, 179], KIF14 is critical for cytokinesis at M phase [180, 181], and CDH-11 can block proliferation and stimulate differentiation [182]. In addition to E2F3, another 6p22 gene, DEK, is also induced in retinoblastoma [14, 17] and its product can promote senescence [183]. Thus, post-***RB*** genetic lesions in human retinoblastoma affect factors that control division.

However, other post-***RB*** defects may target apoptotic regulators. Caspases are proteolytic enzymes that play central roles in apoptosis, and DNA methylation at the caspase 8 locus was detected in 10/17 (59%) primary human retinoblastoma tumors and 2/2 cell lines [184]. Such

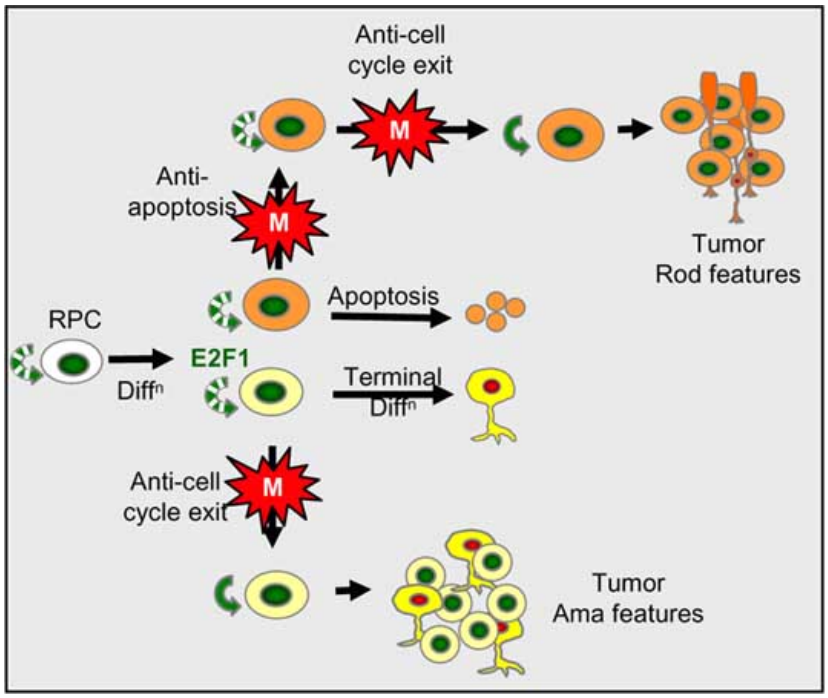

Fig. (2). The cell of origin influences the number and type of mutations required for transformation. In the ***Rb***-deficient retina, RTCs divide due to unleashed E2F1 activity. The green and white striped arrow indicates Rb-deficient RTCs have a finite proliferative capacity. Apoptosis permits a subset of RTCs, including rods, to escape transformation. Another subset, such as amacrine cells (ama), escape by Rb-independent cell cycle exit linked to terminal differentiation. In the latter case, a post-***Rb*** mutation is required to permit permanent division (solid green arrow) and tumor growth. Death-prone cells would require two mutations that block both apoptosis and cell cycle exit.

methylation is common in other pediatric but not adult tumors and is associated with gene silencing in numerous cell lines, including the two retinoblastoma lines [184]. It is not yet clear if this defect is acquired early in tumor progression or if it is a late response. An early mutation would allow death prone RTCs to escape apoptosis. However, mutations in anti-apoptotic regulators could also be late events, well beyond the initial transformation, for example to overcome hypoxia-induced death in already established tumors. Mouse models will be invaluable in testing the role of proteins that regulate either proliferation, such as E2F3, DEK, KIF14 and CDH-11, or apoptosis, such as caspase 8, in retinoblastoma progression, and in deciphering at what stage these factors affect progression.

5. An Explanation for the Existence of Pediatric Tumors?

Overview: ***The demonstrated natural death-resistance of some RTCs as well as theoretical absence of some DNA repair checkpoints in differentiating cells might explain why pediatric tumors like retinoblastoma arise in fewer rate limiting steps than adult tumors.***

Most cancers arise in adulthood, so why do pediatric tumors exist? Statistical analyses of the type Knudson performed indicated that childhood tumors arise in much fewer rate-limiting steps than adult tumors, such as lung, breast or colon cancer. However, the basis for this difference is unknown. One explanation is that the cell of origin of a pediatric tumor has some built in cancer-like properties that make it more susceptible to transformation. As noted above, mouse amacrine cells are naturally death resistant in the face of an oncogenic insult [61]. Resistance to apoptosis is a hallmark of neoplastic cells [185] and, therefore, an ideal property for a cancer cell of origin. The resultant reduction in events required for transformation (Fig. **2**) helps explain the existence of retinoblastoma and possibly other pediatric tumors pediatric tumors.

Could RTCs offer other shortcuts to transformation? Other hallmarks of cancer include genetic instability and invasiveness [185]. Newborn RTCs are meant to be post-mitotic and may down-regulate some repair pathways usually present in dividing cells (such as RPCs). Thus, ectopically dividing RTCs may be more susceptible to genetic lesions. Moreover, Rb plays a variety of roles in maintaining a stable genome [186] (for more discussion see [177]), so ectopically dividing Rb-deficient RTCs could be a hotbed of mutagenesis. With respect to invasiveness, it is intriguing that while many cell cycle genes are deregulated in ***Rb***-deficient serum-starved MEFS, fewer such targets are induced in the absence of both ***p107*** and ***p130***; instead, deleting these genes results in over expression of genes that link the extracellular matrix to growth [43]. The genes that are induced in the human $RB^{-/-}$ human or mouse $Rb^{-/-}p107^{-/-}$ retina cells are unknown, as is the natural invasiveness of these cells.

Cells that naturally provide strong defenses against apoptosis, have reduced defenses against mutagenesis, and are a short step away from being resistant to the usual constraints that cell-cell contact place on multiplication and movement would make an ideal starting point for pediatric cancers that must arise in only a few genetic steps. Animal models will be essential in testing these speculative notions.

6. Is the p53 Pathway Important in Retinoblastoma?

Overview: ***More aggressive mouse models of retinoblastoma are possible in a p53 null background. Whether this indicates that p53 must be inactivated in tumors per se is moot.***

The ***p53*** tumor suppressor is mutated in many human tumors and has diverse functions in cell cycle arrest, apoptosis, genome stability, senescence and cell migration [135, 136, 187]. Remarkably, even though retinoblastoma cell lines can undergo p53-dependent apoptosis [188], ***p53*** mutations have never been found in human retinoblastoma or in tumors from chimeric or conditional ***Rb/p107*** DKO retina [59-61].

This striking negative finding could mean that ***p53*** mutation is lethal or inconsequential in retinoblastoma precursor cells. The idea that it is lethal seems unlikely given that retinoblastoma growth is enhanced in a ***p53*** null background [133, 140]. An inconsequential outcome of ***p53*** mutation could reflect redundancy. The related p63 and p73 proteins are obvious possibilities, and both play important roles in neurogenesis [189]. Multiple splice variants exist, of which the longer versions have similar roles to p53, whereas N-terminal truncated versions appear to act as dominant negative molecules. However, whether p63 or p73 are expressed in the developing retina, whether they are induced in the absence of Rb family members, which splice variants are expressed, and their biological effect is unknown.

Another possibility is that, rather than gene mutations, p53 protein function is inhibited in retinoblastoma by high levels of inhibitors or low levels of prerequisite activators, either naturally in the cell of origin or through mutation. In support of this idea, p53 is sequestered in the cytoplasm of some retinoblastoma cell lines [190]. However, p53 is typically nuclear in primary tumor samples [190]. Some p53 regulators are mutated frequently in human cancer. For example, p53 stability is regulated negatively by HDM2/MDM2 or its relative HDMX/MDMX/MDM4, and positively by $p14^{ARF}$ ($p19^{ARF}$ in mice). MDMX is located at 1q32, a site of frequent gain in human retinoblastoma, although an excellent candidate in this region is KIF14 [15].

Intriguingly, a peptide HDM2 inhibitor induced apoptosis in two retinoblastoma cell lines, and tumor regression in a rabbit xenograft model [191].

It is important to point out that HDM2 and $p14^{ARF}$ also have multiple p53 independent functions. MDM2 binds and regulates several proteins in a p53-independent fashion: it increases HIF1-α levels which permits tumor cells to resist hypoxia [192], prevents E2F1 degradation [193], triggers $p21^{Cip1}$ degradation [194], and inhibits nuclear localization and activity of PML [195]. Many MDM2 variants in human tumors have defective p53 binding domains [196], some of which transform cells lacking p53 [197]. $p19^{ARF}$ also has p53-independent tumor suppressor functions [198] which might include inhibition of ribosome synthesis, blocking activation by the E2F1, Myc and NFκB proteins, stimulating sumoylation and, most recently, inducing autophagy by a small form of ARF generated through initiation at an internal ATG codon [199, 200]. Thus modification of p53 function is not necessarily the mechanism by which other proteins in the p53 pathway facilitate transformation.

Retinoblastoma in ***Rb/p107*** DKO chimeric or conditional models also lack ***p53*** mutations [60, 61]. Yet paradoxically, ***p53*** loss can facilitate mouse retinoblastoma. Occasional cases of retinal dysplasia, but not tumors, are seen in ***$Rb^{+/-};p53^{-/-}$*** and ***Nes-Cre1;$Rb^{f/f};p53^{-/-}$*** mice [148, 163]. Moreover, ***p53*** inactivation is critical for tumors in ***IRBP-E7*** mice [133], and results in more aggressive tumors with higher penetrance in ***Chx10-Cre;$Rb^{f/f};p107^{-/-}$*** mice [140]. These pro-retinoblastoma effects are not related to inhibition of apoptosis because, although indirect hypoxia-driven apoptosis in the ***$Rb^{-/-}$*** embryo is p53-dependent [201], p53 inactivation in photoreceptors does not block apoptosis or facilitate transformation in ***Rb/p107*** DKO chimeras [60], and a ***p53*** null background does not suppress apoptosis either in ***Nes-Cre1;$Rb^{f/f}$*** retina [62] or in ***IRBP-E7*** photoreceptors [134]. Thus, direct inhibition of apoptosis is not the mechanism by which ***p53*** loss triggers or enhances tumorigenesis in ***IRBP-E7*** or ***Chx10-Cre;$Rb^{f/f};p107^{-/-}$*** retina, respectively.

Another possibility is that p53 loss prior to tumor initiation (e.g. in the germ line) increases mutagenesis. Even if ***p53*** mutations were ineffective in ectopically dividing RTCs due to compensation (e.g. by p63 or p73), p53 absence prior to RTC birth could facilitate the accumulation of mutations in RPCs. There is some evidence against this model. First, there is no increase in the frequency of point mutations or small deletions in ***$p53^{-/-}$*** adult thymus, brain, liver or spleen as assessed using indicator mice [202-204]. However, these "Big blue" mice do not detect large deletions amplification, chromosome rearrangements or aneuploidy (for details see [205]). Second, no gross chromosomal abnormalities were seen in ***$p53^{-/-}$*** embryos [206]. However, small clones of cells carrying such abnormalities would not have been detected. There is counter evidence suggesting that p53 loss does increase mutagenesis in the mouse embryo or triggers processes that facilitate mutagenesis. First, ***$p53^{-/-}$*** mice are prone to developmental abnormalities, especially affecting neurogenesis, including eye defects [206, 207]. 5/19 mice exhibited retinal dysplasia with extensive folding [206]. Second, p53 protects against DNA-damaging chemicals and oxidative stress during embryonic development [208]. Third, ***p53*** null mice are more sensitive to ionizing radiation induced tumors and chromosome breaks [209]. Finally, p53 loss provides a proliferative advantage to sub ventricular zone (SVZ) slow and fast cycling RPCs in the brain [210]. Whether this is also the case in retinal RPCs or cells in the early eye field is unknown, but could provide additional opportunities for mutation.

In summary, the relevance of the p53 pathway to human or mouse retinoblastoma is moot. If its inactivation is important, then it must be through p53 regulators. If so, we need an explanation for the complete absence of ***p53*** gene mutations in retinoblastoma, such as compensation by p63 or p73. We also need to clarify whether any defects in p53 regulators really act through p53 and/or one of the many other pathways they target. And we need to explain why ***p53*** mutation in the mouse promotes retinoblastoma even though tumors in Rb/p107 DKO models have normal p53. It does not seem to involve reduced apoptosis, but might involve increased mutagenesis, thus favoring the accumulation of defects that can assist in the full transformation of ectopically dividing RbKO RTCs.

The highly penetrant and more aggressive nature of retinoblastoma in mouse models that include ***p53*** deletion could make them attractive for testing therapies. However, considerable caution is required, as therapies that work by activating p53 and would be effective in treating the human disease, would be impotent in a p53 null mouse and thus discarded incorrectly.

Insight 7: Why Only Humans Develop Retinoblastoma?

Retinoblastoma is extremely rare in any species other than humans [64-66]. Mouse models reveal that resistance is conferred by other members of the Rb family [60-63, 140]. p107 is almost absent during human retinal development, but is abundant in the mouse retina and declines at late stages of development [158, 159]. p130 is present in both human and mouse retina, but is more abundant at later stages [158, 159]. Thus it is not entirely clear why p130 protects against retinoblastoma in mouse but not human retina.

In addition to differences in basal ***p107*** expression, there are also contrasting responses by ***p107*** to ***RB*** loss in mouse or human retina. SiRNA-mediated knockdown of RB in human retinal explants does not affect p107 expression [159]. In contrast, in the ***Nes-Cre1(p)*** model, while p107

protein levels did not change in the E18.5 ***Rb***KO or ***Rb/p130*** DKO retina, and p130 protein levels rose only marginally in the ***Rb***KO or ***Rb/p107*** DKO retina, p107 was more hypophosphorylated, and therefore active, in the absence of Rb, and more so when both Rb and p130 were missing [62]. This post-translational change correlated with decreased cyclin D1 expression, both at the mRNA and protein levels [62].

Rb loss triggers induction of p107 mRNA at later time points [159]. Using explants from ***Rb***$^{-/-}$ mice, no change was observed in p107 immunopositive cells when E13.5 explants were grown for 0 or 4 days ***in vitro*** (DIV), but an increase from 0.3% to 7.5% of cells was seen at 10 DIV (similar to P4.5). In an acute knockout approach, Rb$^{f/-}$ P0 explants were electroporated with Cre plasmid, and an increase in p107 but not p130 mRNA was observed [159]. The effects on p107 protein were not measured in the latter case.

Together, these studies suggest that, in the absence of Rb, p107 protein is more active (hypophosphorylated) at E18.5, likely due to a drop in cyclin D1 levels, and induction of p107 mRNA is limited to later stages of development (after P0). But it remains uncertain as to whether either of these events are important for p107 tumor suppressor activity, since p130 also suppresses mouse retinoblastoma, yet its phosphorylation status or levels do not appear to change in the absence of Rb [62, 159]. This finding suggests that the basal expression pattern of p130 is appropriate to block tumorigenesis without further modification. Tumors in either ***Rb/p107*** or ***Rb/p130*** DKO retina have amacrine cell characteristics, so additional studies are required to specifically address the levels and activity of p107 and p130 in these cells in wild type versus ***Rb***KO backgrounds.

SUMMARY

New models of retinoblastoma suggest that the first critical role of Rb in retinal development is to lock newborn RTCs out of the division cycle. Rb-deficient RTCs continue to divide, and seem a likely starting point for retinoblastoma. Indeed, directed expression of viral oncoproteins proves that RTCs can be transformed. Mouse tumors require inactivation of ***Rb*** and either ***p107*** or ***p130***, yet even in the absence of two of these genes, retina tumors arise sporadically, indicating a requirement for post-***Rb*** mutations. Such defects also seem critical in human retinoblastoma where changes are observed frequently at select chromosomal loci. In the mouse, tumors arise from amacrine transition cells, which are naturally quite resistant to apoptosis. Most amacrine cells escape tumorigenesis not by dieing but by ***Rb/p107***-independent cell cycle arrest linked to terminal differentiation. Thus, after the first hurdle set by Rb (and p107 or p130), post-***Rb*** mutations are required to overcome Rb-independent cell cycle exit. Transformation of death-prone Rb-deficient cell types, such as photoreceptors, would require mutations that bypass both apoptosis and cell cycle arrest***. Rb/p107*** DKO photoreceptor transition cells survive longer when surrounded by normal cells. In humans, where retinoblastoma always arises from sporadic RbKO clones, extended survival of dividing photoreceptors transition cells could provide time for the accumulation of both anti-apoptotic and anti-cell cycle arrest mutations necessary for transformation. Apart from intrinsically high resistance to apoptosis, Rb-deficient RTCs may have other features that accelerate transformation, such as reduced genome stability and/or invasiveness. These predisposing characteristics might explain the existence of retinoblastoma and other pediatric tumors that, unlike adult tumors, only require a few rate limiting oncogenic events.

Animal models will provide a useful platform to extend these discoveries and address numerous outstanding questions. For example: What are the factors that make ectopically dividing RTCs more death-resistant and others more death prone? What are the post-***Rb*** mutations that permit full transformation? What are the survival factors that normal neighbors provide that extend the life of ectopically dividing mutant photoreceptor transition cells? Like death-resistance, are genome instability and/or invasiveness or other cancer hallmarks natural characteristics of Rb-deficient RTCs? If so which DNA repair pathways and factors are involved? Are the features that make RTCs an easier target for transformation important in other pediatric cancer cells of origin?

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