Retinoblastoma: the disease, gene and protein provide critical leads to understand cancer

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Retinoblastoma has contributed much to the understanding of cancer. The protein product of the RB gene, pRB, is a multifaceted regulator of transcription which controls the cell cycle, differentiation and apoptosis in normal development of specific tissues. Elucidating the mechanisms in which pRB plays a critical role will enable novel therapies and strategies for prevention, not only for retinoblastoma, but for cancer in general.

Key words: retinoblastoma / cancer / tumor suppressor / mutation identification

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Introduction

Understanding the molecular basis of the very rare childhood eye cancer, retinoblastoma (RB), has revealed fundamental principles of cancer to an astonishing degree. The disease teaches us that the protein product of the RB gene, pRB, is essential in human retinal development. In the absence of pRB, human developing retina is at extreme risk of forming focal tumors, while certain other tissues are at slightly elevated risk\textsuperscript{1} and yet other tissues show no elevation in risk.\textsuperscript{2} However, pRB is a transcription factor that functions at the core of developmental decisions in cell division, differentiation and apoptosis in almost all cell types. The retinoblastoma disease, gene and protein have all contributed greatly to the concept that cancer can result from derangement of these developmental processes.

Knowing the RB mutation in the child with retinoblastoma has already been shown to have a beneficial impact on the quality and cost of health care for these families.\textsuperscript{3} When we can understand fully the reason for the exquisite tissue specificity of induction of cancer in the absence of pRB, we will uncover opportunities to treat more effectively and prevent retinoblastoma. However, the additional benefits of understanding the role of the RB gene, pRB, and homologous and interacting genes and proteins in normal cell division, differentiation and apoptosis, will be the concepts and strategies to address the broader issue of cancer in general.

Retinoblastoma: the disease

Retinoblastoma is a rare malignant tumor of the developing retina with an incidence of 1 in 20,000 live births in all human races, and this incidence does not vary with geography or level of industrialization. There is no validated documentation of spontaneous retinoblastoma in any other species. Since only 10% of affected children have a family history to warn of the child’s risk, most commonly the tumors are only discovered when one or both eyes is so full of tumor that the pupil appears white, showing a ‘cat’s eye’ appearance. Even at this stage the tumor cells are most commonly contained within the eye and cure is attainable with modern medical care in more than 95% of children.\textsuperscript{4} Salvage of useful vision is possible for moderate sized tumors with radiation or chemotherapy and laser and cryotherapy, and for small tumors with laser and cryotherapy. If retinoblastoma extends outside the eye, mortality is very high.
**Retinoblastoma: the gene**

**The first tumor suppressor gene**

Hereditary retinoblastoma presents as a Mendelian autosomal dominant trait, due to high penetrance resulting from the nearly 100% risk of at least one retinoblastoma forming in those with a ‘null’ RB mutant allele. Despite no family history, most persons with mutant germline RB alleles are easily and certainly identified by the presence of bilateral disease. Alfred Knudson realized the implications of the long known fact that individuals with hereditary bilateral retinoblastoma were diagnosed at a younger age than those children with unilateral, mostly non-heritable, disease. Statistical analyses indicated that as few as two mutational ‘hits’ were rate limiting for the development of retinoblastoma tumors. The occurrence of the first mutation (M1) in the germline and all developing retinal cells gives retinoblastoma tumors a ‘head start’ in hereditary cases (only M2 must arise in a retinal cell), compared to non-heritary tumors where both M1 and M2 must arise in a single retinal cell. That the gene predisposing to retinoblastoma is a tumor suppressor gene, where M1 and M2 are mutations in the two alleles of one gene that is recessive at the cellular level, was suggested by Comings. The RB locus was mapped to chromosome 13q14 by linkage studies and deletion analysis. The proof that the RB gene is a tumor suppressor gene was the observation that the normal allele was often lost (M2), and the mutant RB locus (M1) was duplicated in many retinoblastoma tumors. This loss of heterozygosity (LOH) is evident in 60% of both hereditary and non-heritary RB tumors, manifest by such non-disjunction and duplication of the whole mutant chromosome, or mitotic recombination.

Dryja et al. located a molecular clone at the RB gene locus that was totally deleted from one retinoblastoma tumor. This clone was used to isolate a cDNA that showed sequence conservation among different species. Internal mutations in the gene in retinoblastoma tumors and patients confirmed the identity of the RB gene. The normal allele provides the required functions of RB in the constitutional cells, including the non-malignant cells of the retina, of persons with germline RB gene mutations. The process of LOH is probably occurring in all tissues, yet only in specific tissues is malignancy the consequence of loss of pRB function. The contrast between widespread expression of RB and tissue-specific tumorigenicity could be due either to compensation by other pRB-related proteins, or efficient clearance of certain RB−/− cell types by apoptosis.

**Loss of pRB is insufficient for development of retinoblastoma**

The ‘two-hit’ model for retinoblastoma correctly indicated that at least two events occur before the child shows a tumor. Much evidence indicates that those two events, which result in loss of pRB from a developing retinal cell, are insufficient for malignancy. First, the benign expression of RB gene mutation is a ‘retinoma’, a non-malignant precursor of retinoblastoma. These retinal masses are distinctive and do not change through adult life, but are susceptible to progression to full malignant retinoblastoma.

**Low-penetrance retinoblastoma**

In some families a ‘low-penetrance’ (lp) RB allele results in retinoblastoma tumors in less than 50% of predisposed eyes. The vast majority of high-penetrance mutations are ‘null’ alleles, which destabilize RB mRNA, presumably due to premature truncation of translation, so that no pRB is detectable.

The lp phenotype can result from several different types of RB alleles. First, germline deletion of the whole RB gene often results in unilateral or lp retinoblastoma, presumably because an unknown adjacent critical gene is also deleted, without which the RB−/− cell cannot survive. Only cells in which M2 is a different intragenic RB mutation on an allele with the adjacent critical gene still intact can survive to form retinoblastoma. Second, some mutations...
Figure 1. Low-penetrance (lp) RB mutations cause only 30% of the numbers of tumors as do high-penetrance, null alleles. In the depicted model, a normal allele contributes 10 units of pRB activity. Retinoblastoma tumor occurs when less than 8 units of activity are present. Therefore, LOH for a 4-unit lp allele would not initiate a tumor. Persons with such an allele would only develop tumors when M2 is a separate allele with less than 4 units of activity.

reduce expression of wild type pRB by targeting the promoter or splice sites. Third, in-frame mutations result in a stable pRB with some aberrant functions. In the latter two circumstances, LOH may not result in a tumor because two partially defective lp alleles are thought to express sufficient wild type activity to suppress tumorigenesis, so retinoblastoma arises only in cells where M2 is a separate ‘null’ mutation (Figure 1). Alternatively, two copies of an lp allele with some intact pRB functions and other functions impaired or abolished, may cause apoptosis by creating a signaling imbalance in the developing retinal cell. Low-penetrance mutations target different regions of pRB and provide an opportunity to determine which pRB functions are important for tumor suppression in vitro.

Retinoblastoma: the protein

The word ‘retinoblastoma’ is much better known now for the protein, a cornerstone of the cell cycle, than it is for the disease in children. Before the RB gene was cloned in 1986, about 100 papers/year mentioned ‘retinoblastoma’ in the abstract. In 1999, 698 papers included ‘retinoblastoma’ in the abstract. There has been no increase in the incidence of the disease, but there is an explosion of knowledge of the pivotal role of pRB in development, the cell cycle and cancer.

Although pRB is widely expressed in adult tissues, its developmental expression pattern correlates with differentiation. It is a nuclear transcription factor that is regulated by phosphorylation through the cell cycle, and that interacts with a myriad of proteins in development and differentiation, whereby it plays a critical role in the control of proliferation.

Structural domains

Human RB contains 27 exons within 180 kb of genomic DNA that produces a 4.8 kb mRNA. Within 2.7 kb is encoded a protein (pRB) of 928 residues (921 in mouse) (Figure 2). pRB can be divided into three protease resistant, soluble, structural domains comprised of the N-terminus, R motif, and A/B ‘pocket’. The C-terminus degrades with mild proteolysis and is not structurally well defined.

As mentioned above, most RB mutations abrogate pRB expression. Of the few amino acid substitutions and in-frame deletions that have been observed in RB tumors or other tumors (reviewed by Gallie and Lohmann, also see www.dlohmann.de/Rb/mutations.html), many affect
the A/B pocket, pointing to the importance of this domain for pRB function in the retina and other tissues. This domain is necessary for biological functions, including the regulation of growth and differentiation, and biochemical activities, including transcriptional regulation, and interaction with viral and cellular proteins (reviewed by Kouzarides).

The A and B domains require each other to form a functional repressor motif. The reason for this was made clear by the structural characterization of the A/B pocket by X-ray crystallography: the A domain appears to form a supportive scaffold for the B domain which ensures proper folding and stability.

Missense mutations affecting this A/B interface are tumorigenic, suggesting that its structural integrity is important for pRB function. Several cellular and viral proteins bind the A/B pocket using a conserved LxCxE motif which is shown by crystal structure to bind directly to a highly conserved groove on the B domain.

The C-terminal region is structurally undefined, but is critical for growth suppression and contains a nuclear localization signal and cyclin binding motifs ([R/K]xL) that are important for phosphorylation of pRB. The C-terminal region appears to interact with and regulate the A/B pocket.

The interaction of the C-terminal region with the A/B pocket is strengthened by phosphorylation of C-terminal residues, which disrupt interaction of the A/B pocket with LxCxE proteins. The C-terminal region also binds the oncoproteins c-Abl and MDM2. Deletion of exons 24 and 25 within the pRB C-terminus causes low-penetrance retinoblastoma. The protein expressed by this allele is defective in many, if not all, C-terminal functions. Thus, the C-terminus is an important region for pRB biological activity even though, unlike the A/B pocket, it is unstructured.

The N-terminus is also not structurally well characterized; nevertheless, it has key biological effects because deletion mutants are found in human retinoblastoma. The N-terminus, like the C-terminus, also appears to bind the pocket. However, this interaction may potentiate activity, since several pocket functions are impaired by N-terminal mutations. Thus, the N-terminus may promote an active pRB conformational state. In addition to this putative regulatory function, the N-terminus may have other roles, since it binds several proteins.

**pRB and transcriptional repression**

pRB executes its biological effects by both positively and negatively regulating transcription. Positive gene regulation is associated with differentiation. Transcriptional repression, which is better understood, is associated with inhibition of the cell cycle.

There may be many pRB-repressed genes, since chromatin in RB<sup>−/−</sup> fibroblasts is more broadly accessible to nucleases, implying derepression of transcription, than in wild type cells. Eukaryotic transcription is regulated by three RNA polymerases. The pRB binds directly to components of Pol I and Pol III, inhibiting their induction of rRNA and tRNA genes, respectively. It also inhibits Pol II, which transcribes protein-encoding (mRNA) genes. In this case, however, pRB does not bind to the holoenzyme. Instead, it is recruited to a subset of Pol II-regulated promoters by interacting with activators that bind specific promoters. The major factors that carry out this task are members of the E2F family.

E2F proteins activate genes that are required for DNA synthesis (e.g. dihydrofolate reductase; DHFR) and positive regulation of the cell cycle (e.g. cyclin E) (reviewed by Dyson). E2F proteins perform this function as part of a heterodimeric complex with the related DP family of molecules (DP1–3). E2F activates transcription by recruiting general transcription factors such as TBP and TFIIH, and/or by tethering the histone acetylase CBP to promoters.

Five of the six known E2F family members have a pRB family binding motif embedded in a C-terminal transactivation domain. When bound to this motif, pRB represses transcription by a variety of mechanisms (Figure 3). First, pRB prevents E2F interacting with factors like TBP. This simple competitive effect on an activation domain is termed ‘quenching’ (Figure 3(a)). Second, once tethered to a promoter by E2F, pRB can simultaneously bind the activation domain of another activator [Figure 3(b)]. In this way, pRB quenches two activators at the same time.

Third, the E2F/pRB complex can also inhibit other activators even though they are not pRB-binding targets. This type of ‘direct’ or ‘active’ repression is mediated by pRB (not E2F), since a GAL4-RB fusion protein efficiently inhibits promoters bearing GAL4 binding sites. It may be that pRB carries out this function by recruiting one or more of several corepressors [Figure 3(c)].

Although mutation of E2F sites in some promoters reduces activity during the switch from quiescence to G1, the opposite is true in many cases (reviewed by
Curved arrows indicate the positive effect of activators. The L-shaped arrow indicates the transcription start site. Curved lines ending in a bar indicate repression. (a) pRB binds the E2F activation domain, quenching its activity. (b) pRB binds both the activation domains of E2F and another activator (‘A’), quenching their activity. (c) pRB represses activators it cannot bind (‘B’) by recruiting corepressors, such as HDAC. HDAC uses an LxCxE motif to bind a groove on the pRB pocket that is surrounded by positively charged lysine residues (+).

Dyson\(^66\), suggesting that the repressor function of the E2F/pRB complex has a major biological role. Loss of repression may explain the surprising finding that E2F-1\(^{-/-}\) mice develop tumors,\(^82\) although there are other explanations as well (reviewed by Weinberg\(^83\) and Dyson\(^66\)). Thus, although E2F can function as an oncogene, it is also a tissue-specific tumor suppressor.\(^82,84\)

Many of the corepressors that facilitate active repression by pRB are either enzymes that modify chromatin,\(^76-78,85\) are part of complexes that modify chromatin,\(^81,86-91\) or are homologous to other factors that modify chromatin.\(^79,92\) For example, pRB binds histone deacetylases (HDACs), which remove acetyl groups from lysine residues in the N-terminal tail of histones. The resultant increase in positive charge is thought to enhance histone–DNA interaction, and promote a less accessible chromatin configuration.\(^76-78,93\) A different type of pRB-binding chromatin-modifying enzyme is BRG1.\(^94\) BRG1 and its close relative BRM represent mammalian versions of the ATPases that drive the multiprotein yeast SWI/SNF chromatin remodeling complex. BRG1/BRM can also stimulate certain activators,\(^98\) consistent with the involvement of yeast SWI/SNF in both gene induction and repression.\(^99\) This bivalent activity is common among transcriptional regulators, underscoring the important idea that gene regulation by pRB is highly context dependent.

We see that pRB uses a complex variety of mechanisms and proteins to inhibit transcription. A major challenge for the future is to determine when, and at which promoters, each of these alternatives is exploited.

**pRB and the cell cycle**

The cell cycle consists of DNA synthesis (S phase) and mitosis (M phase) separated by two gap intervals, G1 and G2 (Figure 4). When they are not cycling, cells are in a quiescent phase, G0, and extra- and intracellular signals are required to re-enter the cell cycle. Serum-starved cells in G0 will enter G1 upon growth factor stimulation, and will return to G0 if mitogens are removed prior to a point in late G1. Beyond this ‘restriction point’ (R) cells traverse through S, G2 and M, and will not stop even if serum is removed.\(^100\) Importantly, many of the extracellular and intracellular signals that regulate passage through R converge on the pRB pathway.\(^101\)

The regulation of pRB is by phosphorylation. The protein contains 16 CDK recognition motifs (S/TP) for phosphorylation, seven of which are located in the C-terminus (Figure 2). Hypophosphorylated pRB binds target proteins and arrests cells in G1. This block is relieved by a crescendo of CDK-mediated phosphorylation that begins as cells in G1 approach R, and is abruptly reversed at the end of M phase (Figure 4, reviewed by Mittnacht\(^105\)).

The major targets for hypophosphorylated pRB are E2F and corepressors such as HDACs. E2F–pRB corepressor complexes maintain the silence of genes that are required for progression through R, such as cyclin E. To pass this checkpoint, these repressor complexes are disrupted in two stages by the sequential action of cyclin D- and cyclin E-activated CDKs (Figure 4).

As cells exit G0, cyclin D levels rise causing activation of CDK4/6 and phosphorylation of multiple C-terminal sites on pRB.\(^103-106\) Ser 795 (site 12 in Figure 2) is the first site to be phosphorylated,\(^105\) and is critical for inactivating growth suppression by pRB.\(^104\) The C-terminal region of pRB contains a series of (R/K)xL cyclin docking motifs (Figure 2).\(^54\) The D and E cyclins also contain LxCxE motifs,
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Figure 4. (a) The expression pattern of cyclins D, E, A and B, in relation to the increase in pRB phosphorylation that occurs as the cell cycle progresses. (b) The sequential effect of D-cdk4 and E-cdk2 on interaction of pRB with corepressors and E2F. First, D-cdk4/6 phosphorylates the pRB C-terminus, which interacts with the lysine patch in the B domain of the pocket, dislodging LxCxE corepressors such as HDAC. pRB–HDAC-regulated promoters, such as cyclin E, are induced. The pRB–BRG1 interaction is unaffected, so promoters regulated by this complex (possibly cyclin A—see text), remain silent. Next, E-cdk2 phosphorylates S567 in the A domain of the pocket. This event disrupts the intramolecular interaction between the A and B domains, as well as binding to E2F and BRG1. Now both pRB–HDAC- and pRB–BRG1-repressed promoters are active.

which could theoretically mediate binding to the pRB pocket.107, 108 However, the LxCxE motif is not essential for D1 activity in vivo.104, 109

Following D-cdk4/6 phosphorylation, the increase in negative charge promotes an intramolecular interaction between the C-terminus and a series of positively charged lysine residues (the ‘lysine patch’) that surround the LxCxE binding groove in the B domain of the pocket.56 Associated LxCxE proteins, such as HDACs, are dislodged,56 but E2F and BRG1, which do not use LxCxE motifs to bind pRB, continue to interact with the tumor suppressor.97 Phosphorylation of specific C-terminal residues (sites 14 and 15, Figure 2) is also required to disrupt interaction of pRB with SV40 large T, also an LxCxE protein.110 Removing HDACs is thought to relieve active repression of certain target genes, such as cyclin E. In agreement with this, cyclin E expression can be induced by treating cells with trichostatin A (TSA), an HDAC inhibitor.97 Chromatin immunoprecipitation studies, and expression studies with pRB−/− fibroblasts also suggest that cyclin E is a bona fide pRB target.111, 112 Genes that are required later in S phase, such as cyclin A, remain silent, possibly due to the action of pRB–BRG1 (Figure 4).97

The D-cdk4/6-induced rise in cyclin E levels activates CDK2 and triggers the second step in the pathway, as E-cdk2 phosphorylates a serine located near the end of domain A in the pocket (Ser 567, site 7 in Figure 2). This modification disrupts the intramolecular interaction between the A and B domains of the pocket, and dislodges pRB from both E2F and BRG1 (Figure 4).56, 97 Brown et al.113 showed that repression of E2F by pRB requires the accumulation of phosphorylation on multiple CDK sites, which is consistent with the idea that phosphorylation of site 7 by E-cdk2 is preceded by modification of many C-terminal sites by D-cdk4/6. Furthermore, others have used specific inhibitors to show that phosphorylation by D-cdk4/6 is actually a prerequisite for subsequent phosphorylation by E-cdk2.114, 115 Thus, D-cdk4/6 is required both for the induction of cyclin E levels and for unmasking E-cdk2 target sites on pRB.

Release of pRB from E2F and BRG1 negates all mechanisms of repression. In response, cyclin E levels rise higher, and genes that are repressed by BRG1, such as cyclin A, are induced (Figure 4). The second step releases the pRB checkpoint, and cells can progress into S phase.

The simplicity of this model is appealing, but there are a number of issues that are not yet addressed. For example, although over-expression studies suggest that pRB–BRG1 regulates cyclin A,97 DNA binding studies show that E2F tethers p107, not pRB, to this promoter.112 Knockout mouse studies also suggest that cyclin A is regulated by p107/p130 and not pRB.111 So what are the real in vivo targets of pRB–BRG1? The model, as we have described it, implies that E2F–pRB–HDAC and E2F–pRB–BRG1 regulate separate targets (cyclin E and A, respectively; Figure 4). However, Zhang et al.97 showed that prior to the action of E-cdk2, a trimeric HDAC–pRB–BRG1 complex can be detected. Does this complex serve as a reservoir of pRB corepressor pairs? Or does it actually bind to E2F intact, forming a quaternary complex? Alternatively, does HDAC–pRB–BRG1 target other promoters by an E2F-independent mechanism? And what determines which promoters recruit pRB–HDAC, pRB–BRG1, or HDAC–pRB–BRG1? An additional layer of complexity is suggested by data showing that different promoters are differentially sensitive to repression.
by distinct pRB-associated corepressors.\textsuperscript{78, 80} Finally, pRB and its associated proteins are not the only factors that regulate cell cycle promoters,\textsuperscript{116} so the insights gained have to be placed into a more complex, but realistic, context.

**Links between signaling pathways and pRB**

Growth factors, cytokines and hormones tightly regulate cell division, differentiation and death. Several pathways that connect receptors to intracellular signaling cascades have been identified, but few links have been made between these pathways and nuclear factors. Mitogenic activation via tyrosine kinase receptors, estrogen receptors and G protein-coupled thyrotropin receptors is blocked by the CDK4/6 inhibitor p16 or by anti-cyclinD1 antibodies,\textsuperscript{117} suggesting that all these pathways must inhibit pRB to induce division. In addition, TGFβ inhibits cell growth through pRB by decreasing CDK4 levels,\textsuperscript{118, 119} or by increasing the levels or activity of CDK inhibitors.\textsuperscript{120–124} Finally, pRB is required to inhibit proliferation by blocking ras activity, and this is linked to cyclin D1 expression.\textsuperscript{125} An antagonistic relationship between ras and pRB is conserved in worms.\textsuperscript{126}

A more direct and CDK-independent link between signaling pathways and pRB is suggested by the finding that Raf-1 binds pRB.\textsuperscript{127} The proteins are not associated in G0, but interact 30 min after serum stimulation. Raf-1 phosphorylates pRB in vitro, and both kinase activity and interaction with pRB are required for reversal of E2F repression.\textsuperscript{127} Raf-1 reverses the suppression of colony formation in Saos2 cells by pRB, and this also requires direct interaction. In the latter work the p38 kinase did not co-immunoprecipitate with pRB, but in a later study Fas-induced p38 was shown to phosphorylate and inhibit pRB.\textsuperscript{128}

**pRB, differentiation, immunity, and apoptosis**

A wide range of differentiating and differentiated cell types express pRB during development.\textsuperscript{44, 129} Furthermore, studies with knockout mice and/or tissue culture systems have implicated pRB in the differentiation of erythrocytes, monocytes, neurons, lens fibers, skeletal muscle, adipocytes, keratinocytes, and bone.\textsuperscript{17, 19, 43, 130–137}

One major question is whether pRB stimulates differentiation directly, or simply facilitates differentiation by arresting division. Genetic or functional inactivation of pRB in mice is accompanied by excessive proliferation and apoptosis in many tissues, consistent with a role for pRB in terminal mitosis.\textsuperscript{17, 19, 130, 132–134, 138–140} Inactivation of E2F-1 rescues these defects in the lens and central nervous system (CNS).\textsuperscript{141, 142} again suggesting that the main function of pRB in these cell types is block of cell division. Furthermore, in both cultured cortical progenitor cells, and striatum-derived neural stem cells, pRB absence slows cell cycle exit, but does not affect the eventual proportion of neurons and glia.\textsuperscript{143} Functional inactivation of the pRB family by E1A in cortical neurons induces apoptosis.\textsuperscript{144} However, the pan-neuronal differentiation marker Tα1 α-tubulin is induced normally, and E1A has no effect on terminally differentiated cortical neurons. These data suggest that pRB is required for terminal mitosis, but not for the initiation or maintenance of CNS differentiation.

In the peripheral nervous system,\textsuperscript{132, 141} skeletal muscle\textsuperscript{145} and keratinocytes,\textsuperscript{146} pRB is important for terminal mitosis, but also seems to have a direct role in differentiation, since it binds and stimulates the activity of transcription factors, such as MyoD or c-Jun, that activate cell-specific differentiation genes.\textsuperscript{151, 157, 147} In adipocytes, pRB may be required exclusively for differentiation, but not for cell cycle arrest. When fibroblasts are exposed to adipogenic hormones, division stops independent of pRB, but RB\textsuperscript{−/−} cells fail to differentiate.\textsuperscript{135, 148}

Since pRB has distinct roles in blocking division and promoting differentiation, it may be possible to separate these functions at the molecular level. Indeed, Sellers et al.\textsuperscript{43} showed that some pRB mutants that fail to bind E2F-1 retain differentiation functions, such as induction of a bone marker in Saos-2 osteosarcoma cells and potentiation of MyoD activity.\textsuperscript{45} Further analysis of this type should help clarify the molecular pRB activities required for differentiation in different cell types.

In addition to its effects on cell division, differentiation, and death, pRB also plays a part in regulating the immune response. Major histocompatibility complex (MHC) class II molecules are α and β chain heterodimers that present processed antigens to T-helper cells and activate the cell-mediated immune response.\textsuperscript{149} MHC molecules are constitutively expressed on ‘antigen presenting cells’, including B cells, macrophages, and dendritic cells. However, expression can be induced in many cell types by various stimuli, such as IFN-γ.\textsuperscript{150} RB\textsuperscript{−/−} human tumor lines, and RB\textsuperscript{−/−} non-transformed mouse embryonic fibroblasts are defective for IFN-γ induction.
of MHC molecules. Constitutive MHC class II expression (e.g. in B cells) is pRB independent. MHC class II expression on neoplastic cells has been used to engineer effective tumor vaccines (reviewed by Ostrand-Rosenberg). Inactivating RB may help tumors evade MHC class II induction and subsequent detection and elimination by T cells.

Apoptosis is one of the consequences of entry of a cell into S phase unsupported by proliferation signals and factors. Apoptosis is the common result of loss of pRB or deregulation of E2F. RB−/− mice die in utero from abnormal neurogenesis and erythropoiesis. RB−/− mice show an increase in apoptosis in certain neurons and hematopoietic cells, lens, and early muscle precursors. Dependent on the developmental status or cell type, pRB can protect a cell from apoptosis by controlling S phase entry.

In certain tissues, E2F-1-induced apoptosis is p53 dependent and tumorigenesis occurs when the functions of both pRB and p53 are compromised. Not surprisingly, the three DNA tumor viruses, HPV, SV40 and adenovirus, have independently evolved mechanisms to eliminate both pRB and p53 function.

The pRB pathway in cancer

In view of its important role in alleviating pRB repression, it is not surprising that cyclin D is essential for cell cycle progression in RB-positive cells. In addition, in cells that lack pRB, blocking cyclin D activity has no effect. Suggesting that pRB is the major target for D-CDK4/6. This conclusion is strengthened by studies with the INK4 family of CDK inhibitors, p15, p16, p18 and p19, that specifically bind and block CDK4/6. All members of the p16 family tested so far require pRB to block CDK4/6. In vitro experiments have shown that pRB can block cyclin D activity in cells that lack p16, indicating that pRB is not a specific target for CDK4/6.

The pRB pathway plays such an important role in controlling proliferation that it is frequently (and perhaps always) perturbed in human tumors. Although the developing retina is at nearly 100% risk of forming retinoblastoma when both RB alleles become mutated, only a few other types of tumors, such as sarcoma and melanoma, are also initiated by loss of pRB. However, in many other tumors, pRB is commonly inactivated directly by mutation, by viral oncoprotein, or by alteration of other proteins in the pathway of pRB regulation.

The tumor viruses, human papilloma virus (HPV), adenovirus (E1a), and simian virus 40 large T antigen (Tag), induce quiescent cells to enter and progress through the cell cycle. E7, E1a, and Tag contain the LxCxE motif, required for viral transformation, which allows them to bind the pRB pocket (Figure 2). This action pushes cells past R in late G1 into S phase (Figure 4). E1a and Tag inhibit differentiation even in some terminally differentiated cells. In most carcinomas of the cervix, pRB is intact but functionally inactivated by HPV E7 protein, which uses an LxCxE motif to bind the A/B pocket of pRB.

In other tumors, CDK inhibitors are inactivated, while in others, cyclin and CDK levels are increased by amplification and/or chromosomal translocation (reviewed by Weinberg). Since the major role for the p16 family of CDK inhibitors and D-CDK4 is to regulate pRB, one would predict that a specific tumor caused by mutations in one component of the RB pathway could also arise through alteration of any other member of the pathway. However, this is clearly not the case: RB mutations predominate in retinoblastoma and small cell tumors of lung, and pRB is inactivated by virus in cervical carcinoma, whereas p16 mutations are common in glioma and melanoma (reviewed in Weinberg). This suggests that cell cycle regulation may differ in distinct cell types. Alternatively, members of the pRB pathway may have specific roles in regulating differentiation, death, and/or immune surveillance, all of which affect tumorigenicity. Thus, whether an oncogenic mutation leads to uncontrolled proliferation or a safer outcome (e.g. cell death) will depend on the biological circuitry that the pRB pathway is connected to in a particular cell. Some tumor suppressors may also be redundant in certain tissues where their loss has no effect. Redundancy may also explain species differences. Thus, loss of RB is sufficient to cause retinoblastoma in humans, but inactivation of both p107 and pRB is required in rodents.

Retinoblastoma and retinal development

The RB gene is expressed in all adult tissues, but specific cell types initiate expression of RB at specific developmental times. Preliminary studies indicate
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Figure 5. pRB expression coincides with the initiation of terminal differentiation in the retina. When no pRB is present (RB<sup>−/−</sup>) cell cycle exit fails and proliferation continues, but is balanced by apoptosis, possibly forming retinoma. Malignant retinoblastoma arises when additional mutational events (M3) result in failure of apoptosis to balance the abnormal proliferation. That RB-expression in developing retina initiates as the cells commit to differentiation (Figure 5), but only in a limited subset of the retinal cells is pRB detected, even in adult mammals.<sup>28</sup> Cone photoreceptors strongly express RB, while the rod photoreceptors never express RB. In the peripheral retina, the inner nuclear layer consists of mixed horizontal, amacrine and bipolar cells, and contains a mixture of RB-expressing and non-expressing cells. In the macular region, the inner nuclear layer architecture indicates that bipolar cells do not express RB. Thus, retinoblastoma tumors may arise from a subset of retinal neurons that depend on RB expression for terminal differentiation. This is consistent with the expression of retinoblastoma tumors of cone photoreceptor-specific transducins,<sup>172</sup> but not rod photoreceptor-specific transducins.

The CDK inhibitor protein, p27<sup>Kip1</sup>, which causes activation of pRB to block cell cycle progression, is expressed in a pattern coincident with the onset of differentiation of most retinal cell types.<sup>173</sup> In vitro analyses show that p27<sup>Kip1</sup> accumulation in retinal cells correlates with cell cycle withdrawal and differentiation, and when overexpressed, p27<sup>Kip1</sup> inhibits proliferation of progenitor cells. The histogenesis of photoreceptors and Müller glia is extended in the retina of p27<sup>Kip1</sup>−/− mice, which develop an adult retinal dysplasia due to the displacement of reactive Müller glia into the layer of photoreceptor outer segments. These data could mean that active pRB is required to maintain the Müller cell differentiated state. Although the cyclin-CDK binding domain on p27<sup>Kip1</sup> was essential to promote Müller cell differentiation, inhibition of kinase activity was not.<sup>173</sup> Thus, p27<sup>Kip1</sup> may have non-cell cycle related and pRB-independent effects on retinal differentiation.

Absence of pRB from the developing retina results in apoptosis at the time when terminal differentiation would normally occur, shown by the partial rescue of RB<sup>−/−</sup> mice.<sup>28,134</sup> Apoptosis in the absence of pRB in developing retina was evident from the low proportion of RB<sup>−/−</sup> cells in the retina of mice chimeric for RB<sup>−/−</sup> cells.<sup>174</sup> However, mice chimeric for double knockout of RB and p107 (RB; p107<sup>−/−</sup>) developed retinoblastoma-like tumors.<sup>16</sup> Retinoblastoma can form, but loss of pRB is insufficient for malignancy. Human retinoblastoma express normal p107.<sup>175</sup>

Apoptosis plays a very important role in the development and maintenance of homeostasis in the retina. The exquisite architecture of the human retina that permits accurate function relies extensively on the elimination of extra cells. The specificity of cancer initiation in human developing retina in the absence of pRB may be dependent on the failure of the normal process of retina-specific control of cell numbers by apoptosis. The M3 event (or events) that initiates retinoblastoma may be disruption of the apoptosis pathway (Figure 5). The general process of apoptosis is intact in retinoblastoma cells and many human RB tumors contain wild type p53, but many other components of the apoptotic pathway may be involved.<sup>28,176</sup>

Recent studies suggest an essential role for the neurotrophins such as nerve growth factor (NGF) in mediating retinal apoptosis.<sup>177</sup> Neurotrophins achieve their cellular effects through two types of receptors. Endogenous NGF induces the death of retinal neurons that express the neurotrophin receptor p75(NTR),<sup>178</sup> a member of the tumor necrosis factor receptor family, while expression of trkA receptors results in cell survival. Ligand specificity, interacting factors,<sup>178</sup> and members of the apoptotic pathway downstream from the membrane vary depending on cell types and other genetic determinants.<sup>178</sup>

The potential roles of the apoptosis retinal pathway in the initiation and progression of retinoblastoma remain to be elucidated. It is possible that retinoma represents a limited linear expansion of RB<sup>−/−</sup> retinal cells due to cell cycle progression in the absence of pRB, balanced by apoptosis, while retinoblastoma...
arises when apoptosis fails as a subsequent event (M3) (Figure 5). In theory, understanding this process could lead to improved treatment and even prevention of new tumors in individuals predisposed to malignancy by RB gene mutations.

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