



Understanding pRb: toward the necessary development of targeted treatments for retinoblastoma

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Retinoblastoma is a pediatric retinal tumor initiated by biallelic inactivation of the retinoblastoma gene (*RB1*). *RB1* was the first identified tumor suppressor gene and has defined roles in the regulation of cell cycle progression, DNA replication, and terminal differentiation. However, despite the abundance of work demonstrating the molecular function and identifying binding partners of pRb, the challenge facing molecular biologists and clinical oncologists is how to integrate this vast body of molecular knowledge into the development of targeted therapies for treatment of retinoblastoma. We propose that a more thorough genetic understanding of retinoblastoma would inform targeted treatment decisions and could improve outcomes and quality of life in children affected by this disease.

Retinoblastoma (RB) is the most common primary intraocular malignancy of childhood, affecting roughly 1 in 15,000 children. When diagnosed at an early stage and treated with standard protocols of systemic chemotherapy and focal consolidative therapy, near-complete cure rates are possible, with most patients retaining normal vision in at least one eye (1). If left untreated, however, RB can lead to devastating consequences, including blindness and death, with tumors disseminating throughout the retina, optic nerve, brain parenchyma, and systemically. While early diagnosis and aggressive treatment strategies have made these outcomes rare in developed countries (2), RB remains a potentially devastating disease in developing nations, where intensive chemotherapy and long-term follow-up are not as readily available (2, 3). Standard chemotherapy protocols include two- or three-drug regimens using alkylating and DNA-damaging agents along with cytoskeletal inhibitors, which can be associated with significant toxicities. Systemic carboplatin treatment has been associated with nephrotoxicity and ototoxicity (4, 5), while local treatment has been associated with multiple ocular complications, including reduced motility (6), severe pseudo-preseptal cellulitis (7), and optic atrophy with ischemic necrosis (8, 9), resulting in vision loss. Etoposide treatment has been associated with secondary leukemias (10, 11). Patients with advanced disease require, in addition to chemotherapy, external beam radiation (EBR) and/or enucleation, with the potential result of disfigurement and secondary malignancies (12). EBR is known to cause mid-facial hypoplasia in young patients and increase the risk for development of soft tissue sarcomas, brain tumors, and osteosarcomas, among other cancers (13, 14). Therefore, while treatment of RB is remarkably effective, it is complicated by the extensive side effects of non-targeted chemotherapy agents, radiation, and surgical enucleation (Table 1). Despite extensive knowledge regarding the genetics underlying the development of RB, there has been limited progress in developing targeted treatments for this disease.

***RB1* mutations in retinoblastoma**

RB results from biallelic inactivation or loss of the retinoblastoma 1 gene (*RB1*), located on the long arm of chromosome 13 (13q14.2). Roughly 40% of RB cases are heritable, with *RB1* mutations occurring in the germline and present in all somatic cells, while 60% of RB cases are non-heritable, with mutations arising locally within the developing retina (15). Despite requiring loss of both *RB1* alleles, the genetics of heritable RB demonstrate autosomal dominance, as almost all children who possess one mutated germline copy of *RB1* develop the disease. In heritable cases, the initial mutated *RB1* allele may be known to be present within the family (familial RB, 25% of heritable cases) or it may have occurred de novo in the parental gametes (sporadic heritable RB, 75% of heritable cases) (15). In both cases, mutation of the second allele occurs within the developing retina, resulting in the development of intraocular tumors. In sporadic, non-heritable RB, which comprises the majority of RB cases, both *RB1* mutations occur locally within the affected retina. For this reason, most sporadic non-heritable cases are unilateral, while all bilateral cases have been found to result from heritable germline mutations (15). It remains unknown why the retina is so susceptible to mutation at the *RB1* locus, and an enhanced understanding of why loss of heterozygosity frequently occurs at this locus would improve efforts to both treat and prevent this devastating disease.

The disease burden of patients affected with RB correlates with the mode of inheritance of their mutation. In familial RB, carriers of a null *RB1* allele develop bilateral, multifocal tumors with very high penetrance (90%–100%), while carriers of uncommon partial function mutations more frequently develop “low-penetrance retinoblastoma,” a less severe form of the disease characterized by unilateral involvement, benign retinoma, and decreased penetrance (16). Roughly 10% of children with heritable forms of RB also develop primary midline intracranial neoplasms, most commonly primitive neuroectodermal tumors, a condition referred to as “trilateral retinoblastoma” (17). Patients with heritable *RB1* mutations also have an elevated lifetime risk for developing second primary malignancies, including osteogenic and soft tissue sarcomas, melanomas, and lung and bladder cancers (14, 17–19). These tumors collectively constitute the *RB1* cancer syndrome. Further-

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Table 1
International classification system for retinoblastoma

Group	Clinical characteristics	Treatment (toxicities)	Prognosis
A: Small	≤3 mm height; ≥2 disc diameters from fovea; ≥1 disc diameter from optic nerve	Argon-YAG laser (vitreous seeding if power too high) ^A Diode laser–induced hyperthermia (tissue damage, vitreous seeding) ^A Cryotherapy (retinal tears, chorioretinal atrophy) ^A Brachytherapy (radiation retinotherapy) ^A	Good visual and overall prognosis; usually eradicated
B: Medium	>3 mm height; clear subretinal fluid <3 mm from tumor margin	Vincristine + low-dose carboplatin, up to 6 cycles (neurotoxicity, hyponatremia, nephrotoxicity, ototoxicity) Focal therapy for 2–6 cycles (vitreous seeding, radiation retinotherapy, retinal tears)	Good visual prognosis
C: Confined, medium	Localized vitreous seeding or subretinal seeding or both	Vincristine + high-dose carboplatin + etoposide + G-CSF, up to 6 cycles (neurotoxicity, hyponatremia, nephrotoxicity, ototoxicity, secondary leukemias) Focal therapy (vitreous seeding, radiation retinopathy, retinal tears) Possible subtenon carboplatin (decreased ocular motility, psuedo-preseptal cellulitis, optic atrophy with ischemic necrosis)	Visual prognosis variable
D: Diffuse, large	Diffuse vitreous seeding or diffuse subretinal seeding or both; subretinal fluid >3 mm from tumor margin	Vincristine + high-dose carboplatin + etoposide + G-CSF, up to 6 cycles (neurotoxicity, hyponatremia, nephrotoxicity, ototoxicity, secondary leukemias) EBR (mid-facial hypoplasia, soft tissue and osteosarcoma, brain tumors) Possible subtenon carboplatin (decreased ocular motility, psuedo-preseptal cellulitis, optic atrophy with ischemic necrosis)	Variable visual prognosis; high morbidity from treatment
E: Enucleation, advanced	No visual potential or tumor in anterior segment/ciliary body or neovascular glaucoma or vitreous hemorrhage or phthisical eye or orbital cellulitis-like appearance or involvement of optic nerve or extraocular disease present	Enucleation; prophylactic 3-agent chemotherapy if high-risk features for disease dissemination observed on consensus pathologic evaluation (neurotoxicity, hypnatremia, nephrotoxicity, ototoxicity, secondary leukemias)	High morbidity from treatment; no visual potential

^AFocal therapies. Clinical characteristics associated with classification groups, current treatment strategies, and toxicities of current systemic chemotherapeutic approaches are shown.

more, the predisposition toward secondary tumor development is significantly increased by the radiation therapy used to treat more advanced primary presentations of RB (14).

Since its discovery, pRb, the protein product of *RBI*, has become one of the most studied proteins of all time. pRb plays critical roles in regulating multiple pathways that affect oncogenesis (Figure 1), including cell proliferation, death, and differentiation, through regulation of the cell cycle, apoptosis, senescence, and genome maintenance (20). pRb affects these pathways primarily through direct and indirect suppression of gene and protein expression.

Despite the tremendous progress in determining the cellular function and genetic inheritance patterns of mutations in *RBI*, major questions regarding our understanding of RB as a disease remain, including the function of specific *RBI* mutations and the potential

for tailoring treatments to specific gene and protein defects. Furthermore, despite extensive research, the RB cell of origin remains a subject of continued debate, slowing the development of targeted approaches. This review highlights advances in our understanding of the molecular function of pRb, including its role in the development and progression of RB, as well as the major areas in RB research that remain to be explored, toward the necessary development of more targeted approaches for RB treatment. These targeted approaches should be more effective and less toxic when the myriad functions of pRb are fully elucidated and considered.

pRb function in cell cycle arrest

pRb is a transcriptional cofactor and adaptor protein that functions primarily as a regulator of gene expression, influencing

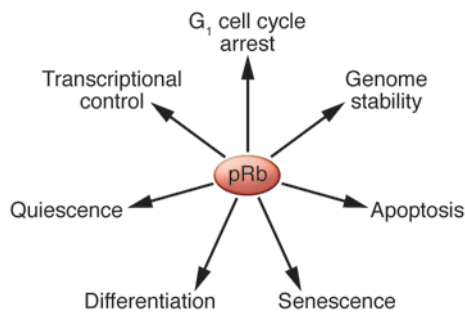


Figure 1
Cellular functions of pRb. pRb is required for regulation of multiple critical cellular processes, including proliferation, differentiation, maintenance of genome integrity, quiescence, and senescence (47, 123).

multiple cell processes required for embryonic growth and development. These processes include cell proliferation, senescence, apoptosis, differentiation, and chromatin remodeling (20). pRb is required for terminal differentiation of myocytes through transcriptional synergy with MyoD, and promotes its functional interaction with the coactivator myocyte enhancer factor 2 (MEF2) (21). pRb also binds directly to histone deacetylase 1 (HDAC1) and prevents it from associating with MyoD, indirectly promoting the expression of MyoD transcriptional targets by preventing their deacetylation (21). pRb also plays important roles in the fate determination, differentiation, survival, and migration of developing neurons (22, 23).

pRb and its related proteins p107 and p130 comprise the family of “pocket” proteins, categorized according to their structural and functional similarities, including the presence of a large pocket domain, which serves as the binding site for many of their interacting proteins. The large pocket of pRb is the site of the majority of naturally occurring mutations in *RB1* and contains distinct sequences required for the binding of two critical mediators of pRb-induced cell cycle arrest, E2F and Skp2 (24–26). While pRb is the predominant pocket protein expressed in murine proliferating postnatal retinal progenitor cells, p107 is upregulated in response to loss of pRb in mice and can at least partially compen-

sate for absence of this protein (27). This compensation has not been observed in humans, which may partially explain why RB is a uniquely human disease.

E2F transcription factors regulate the expression of multiple genes involved in the progression through G₁ and S phases of the cell cycle, in DNA metabolism, and in cell proliferation. pRb binds to DNA-bound E2Fs and inhibits their effects on gene expression by preventing the binding of transcriptional co-activators, and through recruitment of HDACs, ATPases, and DNA methyltransferases to the promoters of target genes (ref. 28 and Figure 2). pRb specifically binds E2F1, -2, and -3 family members (28, 29). E2F1–E2F3 are transcriptional activators and have been found to play an oncogenic role in multiple human cancers, with increased expression levels observed in hepatocellular carcinoma, bladder cancer, glioblastoma, liposarcoma, and breast and ovarian tumors, in addition to RB (30). During the initial stages of G₁, as well as in quiescent or growth-inhibited cells, pocket proteins bind E2F family members to inhibit their transcriptional effects, which would otherwise promote cell cycle progression. The cell cycle regulators under the control of E2F transcription factors include cyclins A and E, Cdc2, Cdc25C, and p21 (ref. 28 and Figure 2).

The F-box protein Skp2 also plays an important role in cell cycle progression, regulating the transition to S phase, as well as cellular senescence, through interaction with the cell cycle inhibitory protein p27. When activated, p27 blocks cell cycle progression through the G₁-S transition by inhibiting the checkpoint cyclin-dependent kinase cyclin E–CDK2 (31–33). The cyclin E–CDK2 complex functions to inhibit pRb activity by phosphorylating pRb and preventing its association with E2F transcriptional activators (ref. 34 and Figure 3A). In the presence of growth-promoting signals, Skp2 binds p27, resulting in p27 degradation. In the absence of proliferative signals, pRb binds Skp2, resulting in Skp2 degradation, subsequent p27 accumulation, and G₁ arrest (Figure 3B). The mechanism underlying pRb/Skp2/p27-mediated G₁ arrest is independent of the pRb–E2F interaction (26), indicating that pRb exerts its antiproliferative, tumor-suppressive, and both pro- (35) and antiapoptotic (36, 37) activities through multiple parallel, though intersecting, pathways. A functional separation between the pRb/Skp2/p27 and pRb/E2F pathways *in vivo* is demonstrated by the presence of *RB1* mutations that inhibit one of these pathways, but leave the other pathway intact.

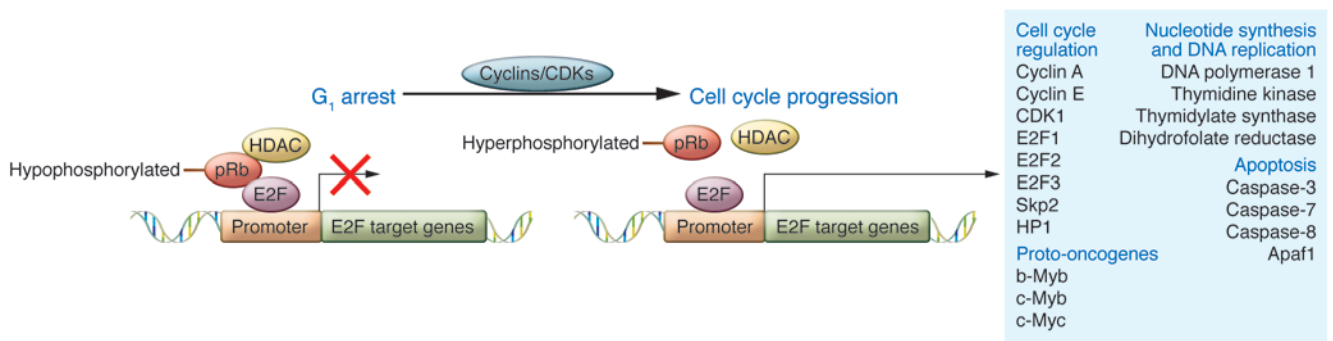


Figure 2
Regulation of E2F-dependent gene expression by pRb. pRb binds to DNA-bound E2F transcriptional regulators and suppresses their target gene expression through recruitment of HDACs, co-repressors, and chromatin remodeling enzymes. It also serves to inhibit the binding of co-activating transcription factors to E2F-bound promoters. Genes regulated by pRb–E2F include regulators of cell cycle progression at the G₁/S transition, enzymes required for DNA synthesis, proto-oncogenes, apoptosis regulators, and modifiers of pRb/E2F pathway activity (47, 57, 124, 125).

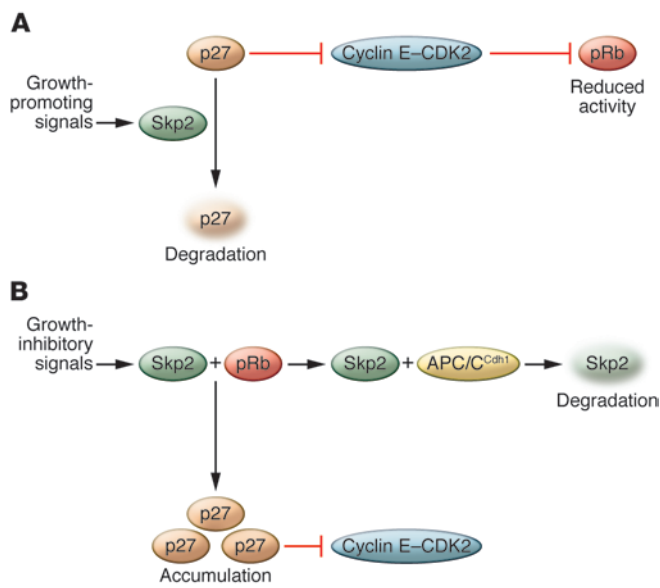


Figure 3

The Skp2/p27/pRb pathway in the regulation of cell cycle progression. **(A)** In response to proliferative signals, cellular levels of Skp2 increase, resulting in Skp2 binding to p27 and targeting it for degradation. This degradation of p27 relieves p27-mediated inhibition of the cyclin E-CDK2 complex during late G₁, allowing for phosphorylation and inactivation of pRb and promoting G₁ progression to S phase. **(B)** In the absence of proliferative signals, pRb is hypophosphorylated and binds to Skp2, preventing it from binding to p27. Skp2 is then complexed with APC/C^{Cdh1} and is targeted for proteasomal degradation. Free p27 is then able to bind cyclin E-CDK2 and inhibit its activity, resulting in continued pRb activity and G₁ cell cycle arrest.

The RbR661W mutation is a naturally occurring partially penetrant mutation of *RB1* that blocks the pRb/E2F pathway, but maintains the pRb/Skp2/p27 pathway (26). Patients harboring the R661W mutation either have unilateral disease or are unaffected carriers (38), which suggests that molecular genetic analysis of all patients with heritable *RB1* mutations may inform treatment decisions, based on the severity of disease and penetrance associated with specific *RB1* mutations.

In addition to E2F and Skp2 binding sites, the A/B pocket of pRb contains a separate binding domain, the LXCXE binding cleft (Figure 4), by which the pRb protein binds to partners that also share an LXCXE binding motif. These include several chromatin remodeling factors that participate in transcriptional repression of E2F target genes, including HDAC1, as well as Cdh1, the partner of APC (20, 39–41). The LXCXE binding cleft is highly evolutionarily conserved and is the target of multiple viral oncoproteins that inactivate pRb to induce tumorigenesis (42, 43). The discovery of these viral oncoproteins led to the development of the first mouse models of RB, in which expression of viral transgenes was targeted to the developing retina. Among these viral oncoproteins are the simian virus 40 (SV40) large T antigen, adenovirus E1A protein, and human papilloma virus E6 and E7 proteins, all of which possess an independently evolved LXCXE sequence (43).

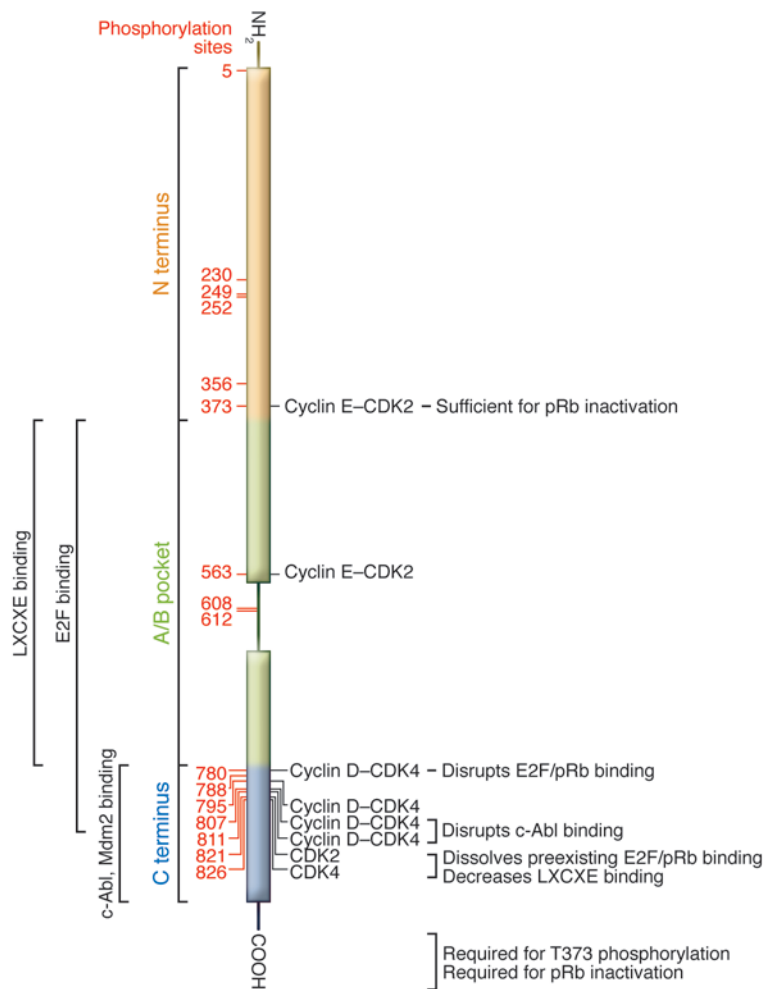
Despite its evolutionary conservation and its function as the binding site for multiple viral oncoproteins, the LXCXE motif of pRb does not appear to serve a critical tumor-suppressive function. Disease-causing mutations in this motif are exceedingly rare and have only been observed in one human cancer cell line (44, 45). Mice genetically engineered to lack this domain are viable and do not develop tumors (46). Interestingly, however, the LXCXE motif does seem to play an important role in regulating the cellular response to toxic exposures, including ionizing radiation and chemotherapeutic agents. This motif has been found to be required to suppress the development of hepatocellular carcinoma following genotoxic stress in mice and to modulate the sensitivity of cultured cells to cisplatin (47). Of note, loss of pRb has also been shown to increase mouse fibroblast sensitivity to etoposide and mitomycin C (48) and increase the sensitivity of

human breast cancer cells to multiple chemotherapeutic agents, including 5-fluorouracil (5-FU), methotrexate, cisplatin, as well as ionizing radiation (49, 50).

Roughly 90% of pRb mutations resulting in hereditary RB are null alleles (16, 51), suggesting that multiple functional domains of pRb contribute to tumor suppression and that tumorigenesis may result from a diversity of mutations that function to globally inactivate pRb (52). Some partially inactivating pRb mutations do occur that confer a lower risk for RB and have provided clues to the discrete tumor-suppressive functions of pRb (16, 53). Mutant forms of *RB1*, such as the aforementioned RbR661W, have been isolated from patients with low-penetrance RB; these mutant proteins have lost the ability to bind E2Fs, but retain their ability to induce differentiation (54, 55). This finding suggests that different regions of the protein are responsible for regulating the diverse cellular functions of pRb and that the nature of the *RB1* gene mutation may dictate disease severity. The elucidation of more precise relationships between specific genetic mutations in *RB1* and the severity of disease phenotypes would therefore pave the way for the development of more targeted patient-specific therapies for RB.

Regulation of pRb activity

The activity of pRb is regulated through control of its phosphorylation state, which is modulated throughout the proliferating cell cycle (56–60). During G₁ and in quiescent cells that have exited the cell cycle, pRb is hypophosphorylated and able to bind E2F and other targets to function as an inhibitor of cell proliferation. In response to proliferative growth signals, pRb is successively phosphorylated by cyclin-dependent kinases (Figures 4 and 5), resulting in its inability to complex effectively with E2Fs (61). pRb is thus released from the promoters of E2F target genes, allowing binding of transactivators and expression of target genes that promote cell cycle progression into S phase (Figure 5). Among the E2F target genes transcribed following pRb phosphorylation is *Skp2* (62), so that both the E2F- and Skp2-dependent pathways are activated to promote cell cycle progression. The pRb protein contains 16 potential CDK phosphorylation sites (ref. 63 and Figure 4), although not all of these have been found to be phosphorylated in vivo.

**Figure 4**

Functional domains of the pRb protein. The A/B pocket, which contains the LXCXE motif, is required for binding of E2Fs, HDACs, and viral oncoproteins. The N terminus contains the phosphorylation site for the cyclin E-CDK2 complex, which is sufficient for pRb inactivation. The C terminus is required for pRb inactivation and contains binding sites for the c-Abl proto-oncogene and the p53 inhibitor Mdm2. The pRb protein contains 16 CDK phosphorylation sites, which alter the binding characteristics and activity of pRb throughout the cell cycle. Mutations in the *RB1* gene are concentrated in the A/B pocket.

Dephosphorylation and activation of pRb is controlled by phosphatases and cyclin-dependent kinase inhibitors (CDKIs) of the Ink4 (p15, p16, p18, and p19) and Cip/Kip (p21, p27, and p57) families (64). CDKIs respond to diverse antiproliferative signals, including DNA damage, senescence, and differentiation signals. Collectively, Ink4 and Cip/Kip CDKIs positively regulate pRb by inhibiting its negative regulators, the activated cyclin-CDK complexes (Figure 5). Inactivation of cyclin-CDKs by CDKIs therefore results in cell cycle arrest in a pRb-dependent manner. Interestingly, despite this highly regulated cycle of phosphorylation, few mutations have been identified that affect pRb phosphorylation, as most highly penetrant mutations result in null alleles (52).

Addition roles for pRb in cell cycle regulation

pRb may also play a role in protecting against the development of chromosomal rearrangements through several distinct mechanisms. Inactivation of pRb results in deregulated expression of the E2F target gene *MAD2*, encoding a mitotic spindle checkpoint protein (65). This results in mitotic defects and aneuploidy in vitro (66), and *Mad2* overexpression has been found to promote aneuploidy and tumorigenesis in transgenic mice (67). Interestingly, *Mad2* is also overexpressed in many tumors, including RB (65), suggesting that this mechanism could contribute to the pathogenesis of the disease.

pRb also ensures chromosomal integrity by directing heterochromatin formation through interactions with two LXCXE-containing histone methyltransferases, Suv4-20h1 and Suv4-2 (68). Disruption of these interactions can result in slowed progression through mitosis and centromere fusion, chromosomal missegregation, and genomic instability (46). pRb regulates chromatin condensation, cohesion, and stability by promoting the centromeric localization of the CAP-D3/condensin II protein complex, an interaction that is evolutionarily conserved (66, 69). These findings suggest that additional mechanisms may underlie the development of RB, including loss of the regulatory interactions between pRb and effectors of normal chromosomal segregation during mitosis. Aneuploidy of chromosomal arms 6p and 1q has been observed in heritable RB tumor samples (70) and has led to the identification of additional genes that might contribute to RB development and progression, as discussed below.

Mouse models of retinoblastoma

Despite the difficulty in generating accurate murine models of RB, much has been learned about the additional pathways required for the development of retinal tumors in animal models. The first heritable model of RB came from a transgenic system in which the SV40 T antigen was expressed under the control of a human leuteinizing hormone beta subunit promoter (LH β -T-Ag).

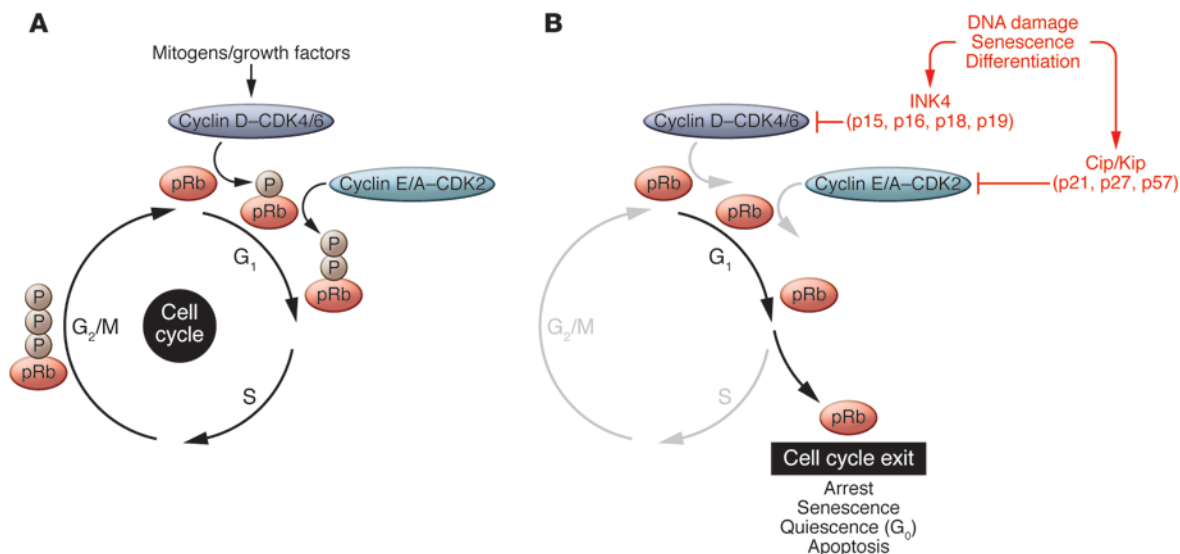


Figure 5

Regulation of pRb in coordination with the cell cycle. Quiescent cells contain hypophosphorylated, active pRb. **(A)** In response to mitogenic signaling, cyclin D-CDK4 complexes phosphorylate pRb and promote its subsequent phosphorylation by cyclin E-CDK2 in late G₁ (28, 126, 127). These phosphorylation events result in inactivation of pRb, allowing progression of the cell cycle past the G₁/S transition and promoting DNA replication during S phase. pRb is further phosphorylated during G₂/M phases of the cell cycle (56, 59, 60). As mitosis comes to completion, pRb is de-phosphorylated and returns to its active, hypophosphorylated state. **(B)** In response to antiproliferative signals (red), CDKIs, including Ink4 and Cip/Kip family members, are activated, resulting in inhibition of cyclin-CDK complexes and promoting pRb activity to cause G₁ arrest and cell cycle exit.

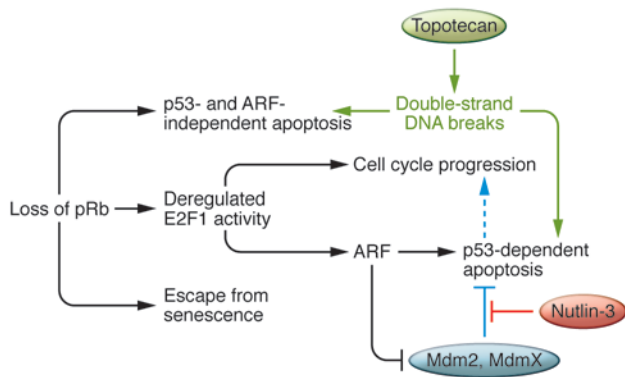
Surprisingly, these mice develop retinal tumors at one to two months of age that are bilateral and multifocal, closely resembling heritable RB (71). The progression of disease displayed by these mice also closely reflected the natural history of human RB, with tumor spreading to invade the choroid, vitreous, optic nerve, and central nervous system. While strong expression of the LHβ promoter in the retina was unexpected, other transgenic models were developed using retina-specific promoters, including those of the interphotoreceptor retinoid-binding protein (*IRBP*), opsin, and alpha-A crystallin genes, which demonstrated varying degrees of disease severity and fidelity to human disease phenotypes (refs. 72–75 and reviewed in ref. 76).

From these models came the critical finding that the p53 tumor suppressor pathway plays an important role in the development of murine RB and may play a significant role in human disease pathogenesis. Expression of the E7 oncoprotein by the *IRBP* promoter results in inactivation of pRb in developing photoreceptors, yet retinal tumors are observed only in mice also lacking p53 (77, 78). Animals expressing wild-type p53 display photoreceptor apoptosis during terminal differentiation, resulting in decreased differentiation and degeneration of the developing retina. While *IRBP*-E7 p53-null mice also display some photoreceptor apoptosis, this apoptosis is p53 independent, with proliferative foci emerging from the degenerating retina (77).

The critical role of p53 loss has been further supported by mouse models of genetic *Rb1* deficiency. Similar to p53 wild-type *IRBP*-E7 mice, mice genetically lacking *Rb1* displayed decreased retinal differentiation and increased retinal apoptosis (79, 80). It is not clear to what extent these ocular phenotypes might be attributable to an extra-embryonic function of pRb, as was demonstrated for other neurologic and hematopoietic phenotypes

observed in these embryos (81). Nevertheless, embryos genetically lacking *Rb1* displayed apoptosis of multiple retinal cell types, including ganglion cells, bipolar cells, and photoreceptors. In contrast to this primarily apoptotic phenotype, chimeric mice lacking both pRb and p107 display retinal tumors that express markers of amacrine cell differentiation, in addition to accelerated retinal degeneration (82). In these mice, photoreceptors committed to differentiation undergo apoptosis, while early retinal precursors form proliferative foci, resulting in a mixed phenotype of aberrant apoptosis and aberrant proliferation. In these pRb/p107-deficient chimeric mice, loss of p53 dramatically accelerates tumor formation. These results suggest both that p107 is able to compensate for loss of pRb in mice and that loss of p53 accelerates tumor formation but is not absolutely required for retinal tumor development.

It is possible that in this model, mice genetically wild-type for p53 undergo functional inactivation of the p53 pathway in the process of developing retinal tumors. In fact, genetic mutations in *P53* have never been observed in RBs isolated from human patients. Rather, human tumor samples display mutations in regulators of p53 (83), suggesting functional, rather than genetic, loss of this surveillance pathway. Additionally, nuclear exclusion of p53, resulting in more invasive portions of RB tumors and in RB cell lines (84). The molecular function of p53 in suppressing retinal tumor development is also still unclear. Inhibition of apoptosis is not observed in the developing retinas of pRb/p107 chimeric mice lacking p53, suggesting that the mechanism underlying accelerated tumor formation is not loss of p53-mediated apoptosis (82). In this system, loss of p53 may accelerate tumor formation through enhanced mutagen-

**Figure 6**

The role of p53-mediated apoptosis following pRb loss. Loss of pRb through genetic mutation results in activation of E2F transcription factors. Deregulated E2Fs can promote cell proliferation through transcription of cell cycle and DNA synthesis genes, but can also induce apoptosis through upregulation of ARF and p53 activity. This control mechanism is lost in the absence of p53. While loss of p53 is rare in RB tumors, amplification of the p53 inhibitors Mdm2 or MdmX (blue) can result in decreased p53 activity, thus inhibiting apoptosis and promoting cell proliferation. Inhibitors of Mdm2 and MdmX, such as nutlin-3 (red), can restore p53 activity to RB tumor cells by preventing association of Mdm2 and MdmX with p53, thus promoting p53-mediated apoptosis. Topotecan (green), another targeted therapy, functions by inhibiting topoisomerase I and creating DNA double-strand breaks, which triggers apoptosis through both p53-dependent and p53-independent pathways (128). Through alternative pathways, deregulated E2F activity resulting from pRb loss can also result in p53-independent apoptosis, as well as escape from senescence and de-differentiation.

esis due to a defective DNA damage response (DDR). This possibility is consistent with the findings that p53 plays a critical role in genomic stability, protecting against chromosomal and DNA strand breakage following ionizing radiation and genotoxic stress, and that mice lacking p53 are prone to tumorigenesis (85–87) and have multiple developmental abnormalities, including retinal dysplasia (88, 89).

Interestingly, deregulated E2F1, which can result from loss of pRb, can trigger apoptosis through a p53-dependent mechanism that results from the accumulation of DNA double-strand breaks (Figure 6 and refs. 90, 91). In response to DNA breaks, p53 activates the DDR, inducing cell cycle arrest mediated by p21 and pRb to allow for repair of the damaged genetic material, or triggering apoptosis if the lesions are not repairable (92). Therefore, in the absence of p53, E2F1-associated DNA damage may not be repaired, increasing mutagenesis and accelerating transformation. However, it is unknown whether activating DDR is actually an important biological barrier to pRb-associated tumorigenesis. Accumulation of E2F1-mediated DNA damage occurs in pRb-null cells, but not in cells in which pRb is functionally inactivated by constitutive phosphorylation (90, 93). This suggests that a checkpoint mechanism may be triggered in response to completely deregulated E2F1, creating strong selective pressure to inactivate p53 (90). In a parallel pathway independent of DNA damage, E2F transcription factors also increase expression of the tumor suppressor ARF, which inhibits Mdm2, an E3 ubiquitin ligase that targets p53 for proteasomal degradation, collectively resulting in p53 activation (Figure 6 and ref. 94).

Associated chromosomal alterations in RB

RB is associated with a discrete set of chromosomal alterations that are frequently seen in combination with mutations of the *RB1* locus. These alterations include +1q, +6p, and -16q, and these chromosomal regions may harbor genes that are critical for RB pathogenesis (95). MdmX, a protein related to Mdm2 that also functions to inhibit p53 (Figure 6), is located in a region of amplification at 1q32, making it a promising candidate as a contributor to RB development. *MDMX* and *MDM2* are both candidate oncogenes for RB development, as the gain or amplification of these genes has been reported in 65% and 10% of RB cases, respectively (83). Amplification of these genes would be expected to functionally inactivate the p53 pathway and may explain the lack of genetic alterations observed at the p53 locus in human RB. However, some reports have demonstrated a lack of correlation between genomic amplification of MdmX and levels of its expressed mRNA (96). Nevertheless, amplification of MdmX has been shown to suppress p53-mediated cell death in retinoblasts lacking *RB1* and to promote their clonal cell proliferation (83). *KIF14*, a putative oncogene and regulator of mitosis, is another candidate gene located in the amplified region of chromosome 1 (97, 98). The expression of *KIF14* is higher than that of *MDMX* in retinoma and RB samples, and expression of this gene is more frequently elevated (99). Nevertheless, complete understanding of the contribution of either of these proteins to disease development and progression requires further study.

Additional candidate genes identified by chromosomal analysis include those encoding the transcription factor E2F3 and the chromatin remodeling factor and histone chaperone protein DEK (98), both of which are located on the amplified region of chromosome 6p. DEK has been identified as a proto-oncogene in multiple malignancies and may be associated with apoptosis inhibition (100–102). Its overexpression is associated with de-differentiation and proliferation (103), in addition to repair of DNA double-strand breaks (104) and inhibition of cellular senescence (101, 105). Other genes proposed as candidates for the progression of RB include the adhesion factor cadherin-11, *P130*, and *MYCN* (95). The precise roles of these genes in retinoblastoma pathogenesis remain unknown.

New treatments based on molecular pathway analysis

Improved understanding of the molecular pathways intersecting with pRb has led to the development of new therapies to better target RB and other tumors harboring *RB1* mutations. Nutlin-3 is a small molecule inhibitor of Mdm2 that also binds MdmX and prevents the association of both of these proteins with p53 (ref. 83 and Figure 6). Treatment with nutlin-3 restores the p53 pathway in RB cells that lack both pRb and p53 activity. The ability of nutlin-3 to inhibit the MdmX-p53 interaction is independent of its inhibition of Mdm2-p53 (83). When used in combination with the p53 inducer topotecan (Figure 6), nutlin-3 functions synergistically to kill RB cells in vitro and results in an 82-fold reduction in tumor burden following subconjunctival injection in mouse models (83, 106). The combination of nutlin-3 and topotecan does not produce any noted side effects in animal models, in contrast to systemic chemotherapeutic regimens. Nutlin-3 is currently in phase I clinical trials for the treatment of RB, with results eagerly anticipated.

Another class of targeted therapies currently in phase I clinical trials for RB are the HDAC inhibitors (HDACi). Cells with elevated E2F1 activity have been found to be uniquely sensitive to HDACi



through overexpression of proapoptotic factors (107). Cells lacking pRb have increased E2F1 activity, and RB-derived cell lines have demonstrated particular sensitivity to HDACi-induced apoptosis (108). In preclinical trials, HDACi inhibited growth of RB-derived tumors in both transgenic and xenograft murine models of RB, with minimal off-target effects (108), suggesting that HDACi may specifically inhibit the proliferation of RB tumor cells and thus have lower systemic toxicities relative to currently used chemotherapeutic agents.

An additional approach would be to target the initial precursor cell from which the tumor derives. The cell of origin of RB, however, remains quite controversial. There is some evidence that RB tumors express markers specific to cone precursor cells, including RXR γ and TR β 2, and the long and medium wavelength (L/M) opsins (109). Furthermore, it has been reported that both cone precursors and RB cells express high levels of Mdm2 and N-Myc, which are required for their proliferation, and that the cone-specific RXR γ induces Mdm2 expression in RB cells in vitro (109), which fits well with data describing loss of the p53 pathway in RB pathogenesis, as discussed above. However, while *MDM2* expression has been observed in RB tumor samples, overexpression of this gene relative to normal retina has not been frequently observed (110). Alternatively, some data suggest that the p53 pathway may be inhibited in RB precursors through overexpression of activating E2F transcription factors, which decrease p53 activity by promoting p53 deacetylation (111). Therefore, while RB tumor cells have some resemblance to cone cells, definitive evidence that cone-specific genes contribute to tumor development is still lacking. The presence of a cone precursor to RB tumors might suggest that these tumors would be more concentrated in the fovea, the portion of the retina with highest cone density; however, this finding is not observed clinically. Other groups have suggested that RB might arise from an amacrine cell precursor, as these cells are the only cells observed to resist death when lacking both *Rb1* and *p107* in mouse models of disease (112). Definitive determination of the cell of origin of RB could promote the development of therapies that specifically target RB cells, while sparing normal retinal tissue, and would shed light on the outstanding question of why retinal cells are so highly dependent on pRb to suppress tumor development.

Localized delivery of non-targeted agents for RB treatment

Despite the identification of multiple potential molecular targets for RB treatment, nutlin-3 remains one of the only targeted agents currently under clinical investigation for this disease. Significant efforts have been employed to investigate the localized delivery of broad-based chemotherapeutics into the ophthalmic artery to minimize toxicities associated with systemic RB treatment. Although reports of this approach have been described for more than 20 years, the currently used technique of intra-arterial injection of melphalan, a DNA-alkylating agent, was first performed in Japan seven years ago (113). While in vitro clonogenic studies showed that melphalan, among 12 chemotherapeutic agents, had the strongest cytotoxic effect on RB cells (114), to date there are no published in vivo data describing the long-term efficacy or toxicities of intra-arterial melphalan in animal models of RB. Through phase III clinical trials in Japan and the United States, intra-arterial melphalan injection has been performed for RB more than 1,400 times (115). Reports have described a tumor response rate between 75%

and 90% and a significant decrease in enucleation rates (116–118). Although there are minimal data regarding long-term follow-up of these patients at present, two-year event-free survival rates have been reported as roughly 70% (118). The lack of long-term survival and toxicity data, from both clinical trials and preclinical animal models, makes the long-term utility of this approach unclear. Complications observed in clinical trials have been serious and have included vitreous hemorrhage, microemboli to the retina and choroid, myositis, eyelid edema, orbital congestion with resulting dysmotility, choroidal atrophy, ophthalmic artery stenosis, and branch retinal artery occlusion, resulting in blindness (119, 120). Although the long-term rate of tumor recurrence or progression to bilateral disease is currently unknown, other treatments delivered by this approach are also under investigation (121, 122).

Genetic testing to inform targeted therapies

Widespread adoption of *RB1* genetic analysis would have a significant impact on treatment decisions and outcomes, without the risks associated with non-targeted approaches. Genetic testing for *RB1* mutations has been limited by the time and costs required for whole gene analysis. The gene lacks known mutation hot spots; therefore, all exons and surrounding sequences must be analyzed individually. However, recently developed high-throughput approaches to gene analysis may allow clinicians and researchers to overcome this obstacle. Whole gene sequencing can directly inform patient care: if an individual who initially presents with unilateral disease is known to harbor a germline mutation, this would argue against enucleation as a primary treatment, because bilateral disease will almost inevitably develop in the future, so systemic chemotherapy would be favored. In contrast, patients who present with unilateral disease and no underlying germline mutation do not have the risk for RB development in the second eye. These infants can therefore be spared the toxicity of chemotherapy or radiation therapy by undergoing enucleation of the involved eye.

Conclusions

Since the discovery of *RB1*, there have been profound advances in our understanding of the genetic and molecular dysfunctions underlying the development of RB. Although the role of pRb in inducing cell cycle arrest and its involvement in regulating cell proliferation, genome integrity, and apoptosis have been extensively studied, the research has led to surprisingly few new targeted therapies for this disease. Similarly, identification of additional discrete genetic lesions associated with loss of *RB1* has not been translated into targeted treatment approaches. We propose that with the development of high-throughput sequencing methods, widespread genetic analysis of *RB1* mutations may provide valuable information regarding disease severity, molecular pathophysiology, and treatment responses for patients with RB. Through this combination of molecular and genetic approaches, the development of more targeted, less toxic approaches to treatment should be achievable for this devastating childhood disease.

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