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# Targeting the RB-Pathway in Cancer Therapy

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## Abstract

The RB-pathway, consisting of inhibitors and activators of cyclin-dependent kinases, the retinoblastoma tumor suppressor (RB), and the E2F-family of transcription factors, plays critical roles in the regulation of cell cycle progression and cell death. Components of this pathway, particularly p16Ink4a, cyclin D1, and RB, are frequently altered in sporadic human cancers to promote deregulated cellular proliferation. The consistent disruption of the RB-pathway in human cancers raises the possibility of exploiting tumor-specific RB-pathway defects to improve the efficacy of current therapies and to develop new therapeutic strategies. This chapter discusses how the RB-pathway status impacts the cellular responses to cytotoxic, cytostatic and hormone therapies, and how the components of the RB-pathway may be directly targeted to treat cancer.

## A. BACKGROUND

### A-1. Definition of the RB-Pathway

The RB-pathway that is discussed in this article consists of five families of proteins (Fig. 1) – CDKN (e.g., Ink4a), D-type cyclins, cyclin-dependent protein kinases (cdk4, cdk6), RB-family of pocket proteins (RB, p107, p130), and the E2F-family of transcription factors (heterodimers of E2F1–7, DP1, 2). This pathway plays a central role in the regulation of cell proliferation as its constituents are activated and/or inhibited by growth-promoting as well as growth-suppressing signals. Furthermore, several components of this pathway, i.e., p16Ink4a, cyclin D1 and RB, are frequently altered in cancer cells including, the deletion/silencing of the p16Ink4a locus, the amplification of the cyclin D1 focus, and the bialleleic mutation of the *RB1* gene. Thus, components of this RB-pathway are rational targets in cancer therapy.

The functional interactions among the five families of proteins in this pathway are well established. The Ink4-family of proteins, p16Ink4a, p15Ink4b, p18Ink4c and p19Ink4d are small heat-stable proteins containing the AKN (ankyrin repeat) domain. Each of the Ink4 proteins can bind to and inhibit the activity of cdk4 and cdk6. The cdk4/6 are D-cyclin-dependent protein kinases. Each of the D-cyclin proteins can associate with cdk4 or cdk6 to form the active kinase complex. The Ink4 proteins compete with the D-cyclins for cdk4/6 to prevent the formation of the active kinase complex. During regulated cell proliferation, the complex of D-cyclin/cdk4/6 is activated as cells respond to mitogenic signals and commit to

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cell cycle entry. The major cellular targets of the D-cyclin/cdk4/6 complexes are the RB-family of pocket proteins, which contain multiple peptide-binding pockets and assemble nuclear protein-complexes to regulate chromatin structures and transcription factor activities. The RB-family proteins are recruited to specific promoters through their interactions with sequence-specific DNA binding proteins. In the pathway discussed here (Fig. 1), the critical interactions are between the RB-pocket proteins and the E2F-family of transcription factors. When recruited to E2F-regulated promoters, RB-pocket proteins inhibit transcription by directly suppressing the transactivation function of E2F and by recruiting factors that mediate transcriptional repression. Phosphorylation of the RB-pocket proteins by D-cyclin/cdk4 and 6 invariably disrupts the RB•E2F interaction, leading to the activation of E2F-regulated gene expression. E2F binds to and regulates the promoters of multiple genes involved in cell cycle progression (e.g. cyclin E and cyclin A), nucleotide biosynthesis (e.g. thymidylate synthase and ribononucleotide reductase), DNA replication (e.g. MCM7 and cdc6), and mitotic progression (e.g. cyclin B1 and cdk1). As will be discussed below, E2F also stimulates the expression of pro-apoptotic genes (e.g., caspases and Apaf-1) (Fig. 1), and thus alterations in the RB-pathway can affect tumor cell response to cytotoxic agents.

### A-2. Alterations in the RB-Pathway in Cancer Cells

Cancer researchers have been interested in the RB-pathway because it is consistently altered in cancer cells to promote deregulated cell proliferation. In this pathway, the Ink4-family and the RB-family proteins function as tumor suppressors, whereas the D-cyclins, cdk4/6 and E2F promote tumor cell proliferation. Recently, a comprehensive analyses of the genome and transcriptome of 206 primary glioblastoma tumors together with the selected sequencing of 601 genes in 91 of the 206 tumor samples have shown that the RB-pathway is altered in 78% of the primary glioblastoma tumor samples. These alterations in the RB-pathway include homozygous deletion and mutation of *CDKN2A* (p16Ink4a) and *RB1* (RB) in 52% and 11% of the samples, respectively, and homozygous deletion of *CDKN2B* (p15Ink4b) and *CDKN2C* (p18Ink4c) in 47% and 2% of the tumor samples, respectively. On the other hand, the *CDK4*, *CDK6* and *CCND2* (cyclin D2) genes are amplified in 18%, 1% and 2% of the glioblastoma tumors examined (1). Taken together, the frequent yet distinct alterations of components of the RB-pathway in cancer raise the possibility for rationally designed therapeutic strategies that exploit defects in this pathway.

### A-3. Role of RB-Pathway in Cellular Responses to Genotoxins

Cytotoxic chemotherapeutic agents and ionizing radiation remain the mainstay therapeutic approaches in the treatment of cancer. These agents almost always cause DNA damage, and the molecular mechanisms underlying the cellular response to genotoxic stresses have been the subject of intense research (2,3). In this context, it is well appreciated that the RB-pathway is regulated at multiple points to instill the appropriate cell cycle inhibition that is induced by DNA damage. For example, cyclin D1 is rapidly degraded following DNA damage (4,5) (Fig. 1). Correspondingly, blockade of cyclin D1 degradation in damaged cells leads to aberrant cell cycle progression that is associated with a breakdown in genome integrity (4,5). The degradation of cyclin D1, together with p53-mediated induction of p21Cip1 and the activity of protein phosphatases cause the dephosphorylation and activation of RB to block cell cycle progression (Fig. 1). The bialleleic loss of *RB1* results in a proclivity of deregulated DNA replication in the presence of DNA damage, and thus resulting in additional secondary DNA lesions and enhanced cellular death (6,7). In addition to cyclin D1 and RB, p16ink4a is implicated in enforcing senescence-like growth arrest in response to the DNA damage (8). Together, several interesting features of the RB-pathway have emerged from these analyses: *First*, the amplification of cyclin D1 does NOT equate with RB loss in the context of DNA damage response, because the overproduced cyclin D1 can still be efficiently attenuated through proteolytic degradation (5). *Second*, RB loss may affect the DNA damage response in

ways that are not found with either the loss of p16ink4a loss or the gain of cyclin D1. In other words, RB function can be controlled by factors beyond those comprising the canonical RB-pathway (9,10). *Thus, in the context of genotoxic response, the individual components of the RB-pathway are important, yet their defects are likely to have common as well as distinct biological consequences.*

### A-4. Role of RB-Pathway in Cellular Responses to Anti-Mitogens

There is a growing class of therapeutic agents that target intrinsic oncogenic or growth stimulatory pathways that are required for tumor maintenance or growth. As such, the plethora of studies that evaluated the importance of the RB-pathway in anti-mitogenic signaling (10, 11) may have applicability to therapeutic agents that perturb these pathways. In general, attenuation of mitogenic signaling results in reduced cyclin D1 levels, limited CDK4/6 activity, and the resultant dephosphorylation/activation of RB (Fig. 1). In this context, cyclin D1 is typically down regulated via a combination of transcriptional and post-transcriptional mechanisms (12). Some anti-proliferative stresses also cause the proteolytic turnover and/or the nuclear exclusion of cyclin D1 (12). Importantly, formation of the cyclin D1-CDK4/6 complex is also dependent on mitogenic signaling. Because of these multiple mechanisms of regulation, the functional impact of deregulated cyclin D1 expression can be highly context dependent.

Unlike cyclin D1 and Cdk4/6, p16ink4a is not generally responsive to mitogenic factors and it is not strongly implicated in the response to antimitogenic perturbations. The loss of RB alone is not sufficient to render cells mitogen-independent, in part due to the activity of the RB-related pocket proteins p107 and p130 that can inhibit cell cycle progression via compensatory mechanisms (13,14). However, the deletion of RB can limit the effectiveness of specific anti-proliferative signals. For example, TGF-beta mediated cell cycle arrest and the anti-proliferative effect of ERK-inhibitors are largely dependent on RB in simple genetic models (15,16). *Thus, there are clear distinctions through which the RB-pathway functions in relation to anti-mitogenic signaling that would be expected to modulate therapeutic response and have implications for clinical response.*

## B. CLINICAL-TRANSLATIONAL ADVANCES

### B-1. RB-Pathway Alterations as Determinants of Therapeutic Responses in Preclinical Models and Clinical Correlates

The findings discussed above from cell culture models have been interrogated in a number of preclinical and clinical settings that have focused largely on two central therapeutic approaches.

**Cytotoxic Agents and Radiation—**As discussed above radiation and the majority of cytotoxic chemotherapeutic agents function at least in part by inducing DNA damage. In keeping with results from model systems, cyclin D1 over-expression is associated with diverse impacts on therapeutic response that seem to be dependent on the preclinical model and the form of therapeutic challenge. However, the number of published studies that interrogated the effects of cyclin D1 over-expression on tumor responses to genotoxins is surprising limited. These studies have found a role for cyclin D1 in either sensitizing to therapy, or compromising therapeutic response (17–20); however, there has not been sufficient validation of these results across patient populations.

Analyses of p16ink4a loss have demonstrated diverse impact of this tumor suppressor on therapeutic response. It may be anticipated that lack of p16ink4a and failure to establish senescence would be associated with poor response to chemotherapy (21); however, this concept has yet to be clearly supported in the analyses of clinical specimens. Interestingly,

some studies have found that elevated levels of p16ink4a in pretreatment biopsies are associated with improved response to cytotoxic therapeutic agents (22–24). Increased expression of p16ink4a is indicative of functional inactivation of RB, as was first appreciated over fifteen years ago in the analyses of cells transformed by viral oncoproteins or harboring RB deletion. In particular, HPV-positive tumors are characterized by elevated p16ink4a staining (24–26), and HPV-positive head and neck tumors exhibit a better response to radiation therapy (24,27). These findings with the p16ink4a-high tumors indirectly suggest that RB inactivation may also contribute to their sensitivity to chemo or radiation therapy.

In breast cancer, lung cancer, and several other preclinical models, loss of RB is associated with increased sensitivity to cisplatin, adriamycin, ionizing radiation, and other genotoxic drugs (28,29). Recent studies suggest that cell cycle deregulation, metabolic stress, and other genetic factors associated with the loss of RB may be particularly relevant co-determinants of tumor responses to cytotoxic drugs (7)(Kay MacLeod personal communication). Because the role of RB in genotoxic response may be context dependent (30), identifying those RB-deficient tumor types that will be particularly sensitive or resistant to cytotoxic chemotherapy or radiation remains a challenge. Nevertheless, in ER-negative breast cancer, RB loss is associated with an improved response to cytotoxic therapy (31). This finding has been further corroborated by gene expression profiling that revealed a statistically significant impact of an RB loss-of-heterozygosity signature on improved therapeutic response (32). In bladder cancer, tumors lacking RB have been shown to display an improved response to radiation (33). The aforementioned radiosensitivity of HPV-positive head and neck cancers, which are defective in RB function, would represent another tumor type supporting the concept (24,27). While these clinical findings are consistent with preclinical studies, it is important to appreciate that RB loss per se may not be the only determining factor, but rather the status of RB may be a marker for a tumor sub-type that is intrinsically more sensitive to genotoxins (32). *Thus, additional studies will be required to clearly elucidate the utility of RB-pathway markers in directing chemotherapy, as this will likely be critically dependent on the tumor type and the therapeutic modality.*

**Hormonal Therapy-Breast/Prostate Cancer—**The hormonal therapy utilized in the treatment of breast and prostate cancer represents one the most commonly prescribed targeted therapies for cancer. In the case of prostate cancer these therapies affect the activity of the androgen receptor (AR), while in breast cancer the estrogen receptor (ER) is the target. Hormonal therapies potently activate the RB function to elicit a cytostatic response. A key factor in the general response to such endocrine therapy is the reduction in cyclin D1 (34,35). In the case of breast cancer this is through complex transcriptional mechanisms (36), while in prostate cancer an mTOR dependent pathway regulates cyclin D1 translation (37). Enforced expression of cyclin D1 has varying impact on the response to endocrine therapies in preclinical models of breast cancer (38–40). Correspondingly, higher cyclin D1 levels are not generally associated with poor prognosis in ER-positive breast cancer (41–44). In prostate cancer, cyclin D1 can function as an antagonist of AR signaling but it is not frequently over-expressed in this tumor type (45).

In contrast to cyclin D1, RB appears to have a potent function in the response to hormonal therapies. Preclinical prostate and breast cancer models demonstrate therapeutic bypass with compromised RB function (34,46–48). In breast cancer, disruption of RB function is associated with a subtype that exhibits poor response to Tamoxifen and related agents, and a benefit of adjuvant chemotherapy (31,32,46). A number of response-predictive gene expression profiles, such as OncotypeDx (49), actually measure RB activity because they interrogate the expression levels of proliferation associated E2F-regulated genes. While the analyses of prostate cancer specimens has been more limited, RB loss was observed progressively in castrate resistant prostate cancer (50). *These combined findings suggest that knowledge of RB-pathway*

*perturbations and the regulation of RB function in breast and prostate cancer may contribute to more effectively deployed hormonal therapies to ameliorate the rate of recurrence.*

### B-2. Novel Therapeutic Approaches—Targeting the RB-Pathway Tationally

There are currently two distinct approaches that are gaining traction in directly targeting the RB pathway to therapeutic effect.

**Taking Advantage of RB-pathway Deregulation—**The concept of exploiting the loss of RB and the consequential deregulation of E2F-activity to kill tumor cells (Fig. 1) was first demonstrated by the result that agents with the capability of aberrantly stimulating E2F activity can elicit increased cytotoxic response in tumor cells with perturbations in the RB-pathway (51,52). Subsequently, a number of groups have explored pathways or agents that have specific activity against the RB-pathway. From these analyses a few different concepts have begun to emerge. *First*, there are cellular signaling pathways that can protect cells with deregulated E2F-activity from death (53,54). Inhibitors of these pathways, by definition, could be "synthetically lethal" with disruption of the RB-pathway in cancer cells. *Second*, the pro-apoptotic activity of p53 is restrained by the RB-pathway (55). As such, RB-deficient tumor cells could be sensitized to p53-mediated cell death. In keeping with this concept, it has been shown that RB-deficient tumor lines or those exhibiting deregulated E2F activity are more sensitive to compounds that have the capacity to activate p53 (56). *Third*, because the RB-pathway impacts E2F transcriptional activity, oncolytic viruses have been produced that would specifically replicate and kill tumor cells that harbor deregulated E2F activity (57).

**Activating the Tumor Suppression Function of the RB-Pathway—**When *RB1* was first cloned over 20 years ago, it was hoped that the discovery of the first tumor suppressor would lend itself to therapeutic approaches for retinoblastoma and additional tumor types. In keeping with that general concept, the enforced expression of p16Ink4a or of constitutively active alleles of *RB1* potently inhibits cellular proliferation and can induce persistent cell cycle arrest that is characterized by certain molecular elements of senescence (58,59). Correspondingly, gene-transfer approaches that specifically target the RB-pathway have been deployed in preclinical models, but not yet in the clinical setting (60,61). It is well appreciated that re-activating compromised tumor suppressors is a significant pharmacological challenge. In terms of the RB-pathway the closest approach to achieving this goal is the use of inhibitors of DNA methylation or histone deacetylases which although cytotoxic and leading to numerous other endpoints, can also lead to the activation of epigenetically silenced p16Ink4a (Fig. 1) (62).

Another means to re-activate RB function in tumor cells is through the use of CDK-inhibitors that would prevent RB phosphorylation and maintain efficient transcriptional repression (Fig. 1). First generation compounds such a flavopiridol, did result in the blockade of RB phosphorylation, and the RB status did play a role in the response to flavopridol (63). However, such compounds are relatively toxic due to effects on CDKs that are involved in the regulation of transcription (64). As such, whether these compounds exert their primary effect through the RB-pathway remains an open question. Second-generation CDK4/6 inhibitors have been developed that are highly selective and seemingly have no off-target effects based on biochemical and cell based assays (64–66). Consistent with expectation, such agents are dependent on the presence of RB for therapeutic effect in preclinical models (66). Of these agents the Pfizer compound, PD-0332991 is the most broadly deployed in the clinic. The Phase I trial of PD-0332991 represented the first targeted use of an agent that specifically activates RB in the clinic. In keeping with the preclinical data, the Phase I trial utilized RB-deficiency as an exclusion criterion, and several Phase I/II single-agent or combination trials are currently in progress (cancer.gov/clinicaltrials). A key question for such highly specific cytostatic agents

is whether they will have therapeutic efficacy, because tumor evolution may readily develop bypass mechanisms to overcome such a single agent. While more trials will be required to answer this question, the preliminary indication from the Phase I trial is that there exist certain tumor types wherein PD-0332991 or related means to chronically activate RB will be therapeutically effective. Specifically, in malignant teratoma continual treatment with PD-0332991 has prevented progression of the disease in patients for approximately two years (67). *Together, these analyses suggest that RB-pathway activation could be utilized therapeutically.*

**Key questions moving forward—**As discussed above there is promise in considering the RB-pathway as a node upon which to make treatment decisions or deploy specific therapeutic approaches. However, in spite of substantial preclinical work and analyses of clinical specimens, a number of impediments preclude the implementation of these ideas broadly in the clinic. *First,* there remains considerable uncertainty about the functional importance of the RB-pathway in reference to therapeutic response in multiple tumor types. *Second,* the means for monitoring RB-pathway function in biopsy specimens is not standardized. *Third,* there have yet to be any study that has demonstrated the ideal means to therapeutically target distinct RB-pathway lesions. *Fourth,* the overall efficacy of prolonged RB-pathway mediated tumor-stasis as a therapeutic option remains uncertain. The address of these issues will require substantial additional studies, and critical re-evaluation of the extensive literature to develop idealized strategies for exploiting the wealth of knowledge in this pathway for the benefit of the cancer patient.

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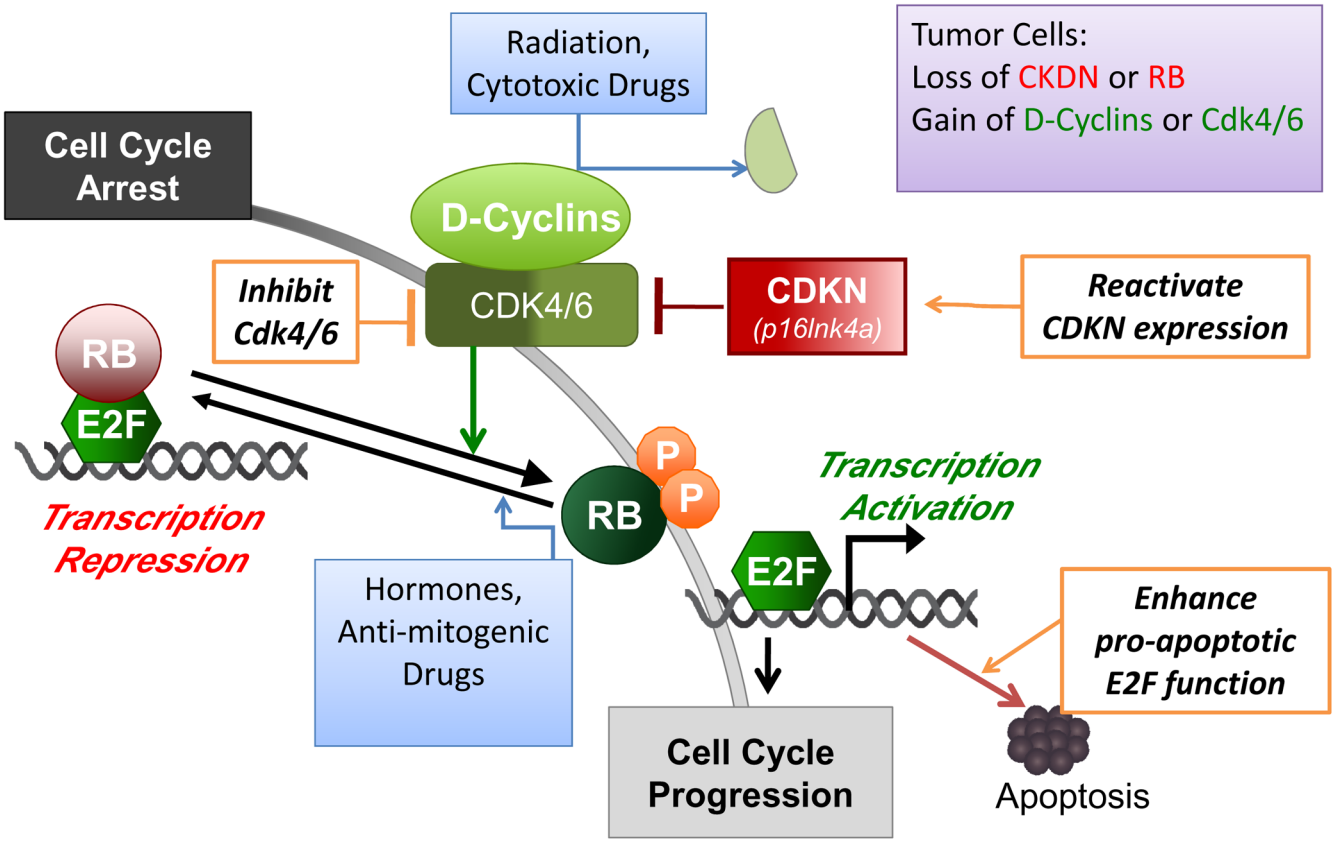


**Figure 1. The RB-Pathway in Cancer Therapy**

The components of the RB-pathway, i.e., RB, E2F, D-type cyclins, Cdk4/6, p16Ink4a (CDKN2a) and their functional interactions, are depicted in the diagram. Genetic and epigenetic alternations in the RB-pathway are consistently detected in the majority of sporadic human cancers, and these defects are summarized in the purple box at the upper right-hand corner of the diagram. The status of the RB-pathway affects tumor cell responses to radiation and genotoxic drugs, which cause cell cycle arrest through the degradation of cyclin D1 and the consequent RB dephosphorylation. The status of the RB-pathway also affects tumor cell responses to hormone and other therapeutic strategies that block mitogenic signaling. Defects in the RB-pathway cause deregulated E2F activity, which stimulates gene expression to promote G1/S transition and apoptosis. Potential therapeutic strategies that directly target the RB-pathway defects are depicted in the diagram in orange boxes, and these include the reactivation of p16Ink4a expression in cases where the gene is silenced but not mutated, the inhibition of Cdk4/6 kinase activity, and the enhancement of E2F-dependent apoptosis.