



Published in final edited form as:

Nat Rev Mol Cell Biol. 2013 May ; 14(5): 297–306. doi:10.1038/nrm3567.

Molecular mechanisms underlying RB protein function

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Abstract

Inactivation of the RB protein is one of the most fundamental events in cancer. Coming to a molecular understanding of its function in normal cells and how it impedes cancer development has been challenging. Historically, the ability of RB to regulate the cell cycle placed it in a central role in proliferative control, and research focused on RB regulation of the E2F family of transcription factors. Remarkably, several recent studies have found additional tumour-suppressor functions of RB, including alternative roles in the cell cycle, maintenance of genome stability and apoptosis. These advances and new structural studies are combining to define the multifunctionality of RB.

The *RB* gene was cloned more than 25 years ago¹. Since that time, its encoded protein has been identified as a universal cell cycle regulator with a central role in controlling the commitment of a cell to initiate DNA replication and divide². Eliminating RB function allows unregulated cell cycle progression and promotes tumour growth. The prominent role that RB has in blocking proliferation has created confusion in understanding its biochemical function. Because of this key function, most upstream events that influence cell proliferation ultimately also affect RB function. However, it is often difficult to distinguish signalling mechanisms that directly impinge on events in the G1 phase of the cell cycle to arrest proliferation from those that act during other cell cycle phases and indirectly cause a G1 arrest. In the context of physiological events that have input into the cell cycle, such as cellular differentiation, cell senescence and the response to DNA damage, it can be unclear how directly signalling pathways actually regulate RB. For these reasons, the ongoing debate on RB function ranges between two extreme views. One view is that RB is a multifunctional protein acting in response to numerous stimuli to create at least as many potential outcomes. The alternative viewpoint is that signals from many different stimuli are ultimately channelled through cyclin-dependent kinase (CDK) regulation of RB phosphorylation, which in turn controls the activity of E2F transcription factors, a family of

Note added in proof

A recent paper also describes a phosphorylation-dependent change in RB conformation that is relevant for understanding how RB interactions with other proteins are regulated⁹².

Competing interests statement

The authors declare no competing financial interests.

transcriptional regulators that stimulate proliferation. Both views have merit, and ultimately most models of RB function fall somewhere in-between, as researchers work to pinpoint its precise function.

Most cancers find a way to impair RB function, either through direct mutation of the *RB* gene or, more commonly, through the altered expression of RB regulators, which include cyclin D, CDK4 and CDK6, and their principal inhibitor, p16 (REF. 3). Given that loss of RB function is frequent in cancer, understanding its mechanism of action is expected to offer crucial insight into the most fundamental properties of cancer cells. Furthermore, loss of RB function in insects and mammals alike leads to overproliferation of cells and defects in numerous stages of organism development. From this perspective, knowledge of RB function promises important advances in our understanding of cancer, as well as of how proliferative control is coordinated in development. These promises can only be realized through a mechanistic understanding of the intricacies of RB function itself.

Much of our molecular knowledge of RB was established using cancer cell lines with aberrant RB pathway function, or was developed before the advent of RNA interference technologies that allow for loss-of-function analyses. The tools to address outstanding questions about RB structure and function in normal cells have only recently emerged. Our goal in this Review is to highlight these exciting recent advances in the biochemical understanding of RB. We describe the structure of RB and how its function is regulated. In particular, we discuss the role of key interacting protein partners that can be placed in the context of a few regulatory pathways for which detailed biochemical information is available. Readers who are interested in how these mechanisms are connected to broader questions of development and homeostasis^{2,4}, as well as cancer initiation, progression^{5,6} and therapy^{7,8}, are directed to other recent reviews specializing in these topics.

RB is a platform for multiple protein contacts

Although recent research has revealed that RB functions in diverse cellular pathways, such as apoptosis and the cell cycle, it has also become clear that RB regulates these pathways through the stimulation or inhibition of the activity of interacting proteins. Therefore, an important starting point for understanding RB function is its structure, which acts as a scaffold for these multiple protein interactions (FIG. 1).

Human RB contains 928 amino acids and is commonly described as having three domains. The central domain was identified as the minimal region necessary to bind viral oncoproteins, such as adenovirus E1A, SV40 TAg and human papilloma virus E7, and it was named the ‘pocket’ (Ref. 9). The pocket comprises two subdomains, A and B, each resembling a cyclin fold with three additional helices¹⁰ (FIG. 1a). The A and B subdomains interact with each other through an extensive non-covalent interface such that the pocket folds into a single structural unit. Two additional cyclin folds constitute the structured amino-terminal domain (RBN), which resembles the pocket structure except for a few subtle differences¹¹. Approximately the last 150 residues of RB form the carboxy-terminal domain (RBC), which is intrinsically disordered¹². There are several other sequences in RB that similarly lack structure, including insertion loops in the RBN and the pocket, as well as a

linker that flexibly tethers these two structured domains (FIG. 1b). The linker sequences are notable because they contain CDK-dependent phosphorylation sites (BOX 1) that have a critical role for the inactivation of RB^{13,14}. RB inactivation through phosphorylation and reorganization of its structure is depicted in FIG. 1c (see below). The domain structure of the RB family members p107 (also known as RBL1) and p130 (also known as RBL2) is similar, which is consistent with their analogous functions in regulating growth, the cell cycle and E2F function (BOX 2).

Evolutionarily conserved interaction surfaces on RB

The modular domain organization of RB, like that of many signalling proteins, allows for independent binding and regulation of multiple protein interactors (FIG. 1). However, unlike typical signal transducers, RB does not contain canonical protein–protein interaction domains (for example, Src homology 2 and phosphotyrosine-binding domains) and instead uses the cyclin folds and the disordered RBC to form binding interfaces. To inhibit transactivation, RB binds the E2F transactivation domain (E2F^{TD}) using a highly conserved region of the pocket domain, which lies at the interface between the A and B cyclin folds^{15,16}. Also in the pocket domain, an ‘L-X-C-X-E-binding cleft’ (in which X represents any amino acid) formed by three helices of the second cyclin fold binds an L-X-C-X-E sequence found in viral proteins such as TAg and E7 (REFS 17,18). This interaction is required for the cellular transforming activity of these proteins. The conservation of this surface in RB orthologues from other species, as well as in its related family members p107 and p130, suggests a crucial cellular role (BOX 2). Importantly, a number of RB–protein interactions have been mapped to the L-X-C-X-E-binding cleft^{19,20}. Although it has been common to use the L-X-C-X-E sequence motif as a consensus for identifying cellular RB-interacting proteins, it has become clear from structural studies with viral and cellular proteins that there are other determinants of high-affinity interactions with the pocket cleft²¹. Furthermore, several proteins that contact this region of RB do not contain a clear L-X-C-X-E motif^{22,23}, and there is little evidence that these or other L-X-C-X-E-containing cellular proteins bind RB directly. Together, these observations indicate that RB interactions with cellular proteins at the L-X-C-X-E-binding cleft are probably more complex than understood from the crystal structures of RB bound to viral proteins. In light of the recent discovery of small molecule therapeutics that can target the cleft²⁴, further structural studies to understand these subtleties are required.

Interactions through intrinsically disordered regions

RB binds several proteins using sequences in the RBN and RBC. The RBN binds E1A-like inhibitor of differentiation 1 (EID1), along with several other proteins, although data to map protein interactions in detail are limited for this domain of RB. A second RB–E2F interaction is made between the RBC and the ‘marked box’ domains of E2F and its heterodimerization partner differentiation-related polypeptide (DP)¹² (FIG. 1). Although the RBC is intrinsically disordered, approximately 30 residues adopt a strand–turn–helix conformation on binding the marked box region of E2F–DP. At least part of this second interaction seems to be specific between RB and E2F1 *in vivo*, and it may be important for the function and regulation of an E2F1-specific activity, such as apoptosis^{25,26}, which is discussed below.

The RBC is also the location of kinase and phosphatase docking sites^{27–29}. These enzymes bind short, linear peptide sequences distal to their target RB phosphorylation sites, and this increases enzyme efficiency by a stronger substrate interaction. Distinct binding sites in the RBC are accessed by cyclin A–CDK2, cyclin D–CDK4 and cyclin D–CDK6, although the implications of this difference for CDK-site preference by these enzymes is not clear^{27,29}. A recent structural study of protein phosphatase 1 (PP1) bound to the RBC demonstrated that the phosphatase and cyclin A binding sites overlap and that PP1 can inhibit CDK activity towards RB through a docking competition mechanism²⁸. RB binds PP1 in a manner that resembles other PP1 regulatory subunits, and it will be interesting to learn whether RB also functions in targeting PP1 to specific substrates for dephosphorylation.

Cell cycle control through E2F regulation

Initial studies of RB focused on its inhibition of E2F transcription before the G1 to S phase transition, as the observed negative regulation of the cell cycle offered a powerful explanation for why RB is a tumour suppressor^{2,30}. These early experiments correlated RB function with its capacity to physically associate with E2F family proteins and thereby inhibit E2F-dependent activation of genes that stimulate DNA synthesis and cell cycle advancement. When RB is in a hypophosphorylated state, pocket–E2F^{TD} contacts and interactions involving the RBC are required for E2F inhibition^{31,32}. CDK-dependent phosphorylation, which occurs on approximately 13 conserved consensus sites *in vivo*³³, inactivates RB-dependent E2F repression by dissociating the RB–E2F complex³⁰ (BOX 1).

Regulation of RB–E2F binding

Recent studies have characterized the structural changes in RB that result in binding inhibition^{12–14} (FIG. 1c). Phosphorylation induces interdomain associations that occlude, or allosterically disrupt, the E2F^{TD}-binding site in the pocket and the interaction between the RBC and the E2F marked box. An important result of this is that discrete phosphorylation events result in distinct RB structures. Ser608 and Ser612 phosphorylation stabilizes the association of the pocket loop with the E2F^{TD}-binding site within the pocket domain. Thr373 phosphorylation induces interdomain docking between the RBN and the pocket, which allosterically inhibits E2F^{TD} and directly inhibits binding at the L-X-C-X-E-binding cleft. Finally, Thr821 and Thr826 phosphorylation induces RBC binding to the pocket, which excludes the E2F marked box and L-X-C-X-E interactions. It is possible that these phosphorylation-dependent conformational changes can control RB interactions with other proteins as well as E2F. Mutational analysis of CDK phosphorylation sites on RB suggests that a large number of sites need to be phosphorylated for RB inactivation and cell cycle advancement^{34,35}. However, there are few data available to indicate the quantity of sites that are phosphorylated on each individual RB molecule during G1 to S phase transition. For this reason, models such as the one depicted in FIG. 1c require further testing, and it is possible that partial phosphorylation could create different functional outputs depending on which specific sites are phosphorylated.

Unlike many other cell cycle regulatory proteins, RB is not typically degraded upon inactivation but persists until mitosis, when it is dephosphorylated by PP1 to enter the following G1 phase³⁶. This presence throughout the cell cycle allows reactivation by

dephosphorylation in response to S phase and other checkpoints. Several recent studies have found that other post-translational modifications, including methylation and acetylation, can control RB activity by regulating RB phosphorylation levels³⁷ (BOX 1).

Chromatin structure regulation at E2F target promoters

RB interactions with chromatin-regulating enzymes and histone-modifying enzymes have been identified and are mediated through the L-X-C-X-E-binding cleft¹⁹. The physical separation between the E2F and L-X-C-X-E-binding surfaces in RB supports a model whereby RB recruits chromatin regulators to E2F-regulated promoters for repression or activation of target genes (FIG. 2). CDK phosphorylation disrupts RB interactions with these proteins by inducing conformational changes that inhibit binding at the L-X-C-X-E-binding cleft and E2Fs^{12,14,38,39}. Mutations in the L-X-C-X-E-binding cleft do not impair the ability of RB to regulate the G1 to S phase transition and cell growth⁴⁰, so the significance of chromatin-modifying enzymes in RB regulation of E2F-controlled gene expression in cycling cells is not clear. The action of these proteins may be more relevant for changes in chromatin structure associated with permanent gene silencing observed in cell cycle exit. Supporting this idea, a crucial role for RB–L-X-C-X-E interactions has been observed in the formation of heterochromatin in senescence⁴¹, and RB and its homologues p107 and p130 contribute to the response of upstream signals that induce senescence and maintenance of cell cycle exit^{42–44}. Phenotypically, these results indicate an important role for RB in recruiting chromatin regulators; however, biochemical demonstration of these complexes remains rare. Recent work has now demonstrated the RB-dependent recruitment of histone deacetylase enzymes using its L-X-C-X-E-binding cleft at E2F target genes by chromatin immunoprecipitation assays⁴⁵.

Transcriptional repression by other RB-family proteins in G1

RB belongs to a family comprising three homologous proteins that are referred to as pocket proteins (BOX 2). p130 is the most abundant pocket protein in stable cell cycle arrest events such as quiescence and senescence^{46,47}. When bound to E2F4, it represses genes required for cell cycle re-entry under both conditions^{48,49}. DREAM, a complex containing p130, E2F4–DP and the highly conserved MuvB complex, was recently shown to facilitate this p130-dependent activity^{49,50}. In human cells, chromatin immunoprecipitation assays showed that MuvB proteins are located at most E2F-regulated promoters, and gene expression analysis has implicated DREAM as a repressor of cell cycle genes. Few details are known about the biochemical function of MuvB, with the exception that it acts as a scaffold for the recruitment of different transcription factors. DREAM is regulated by dual specificity Tyr-phosphorylation-regulated kinase 1A (DYRK1A) and CDK phosphorylation⁵¹. Upon cell cycle entry MuvB dissociates from p130 and binds Myb-related protein B (MYBB; also known as MYBL2) and forkhead box M1 (FOXO1)^{52,53}. These two transcription factors, which are upregulated in many cancers, then activate late-stage cell cycle genes⁵³.

Putting RB–E2F regulation into perspective

The canonical model of RB tumour suppression is based on the negative regulation of E2F transcription⁵⁴. However, p107 and p130 also repress E2F transcription and inhibit the cell cycle, yet only RB is commonly mutated in cancer⁸. For this reason, some cancers may

retain the ability to restrict E2F activation. Two recent studies have also shown that activator E2Fs (E2F1, E2F2 and E2F3) are dispensable for proliferation *in vivo*^{55,56}. From this perspective, it is difficult to envision how RB inhibition of these particular E2Fs can arrest proliferation when they are not required for proliferation. These results suggest that transcriptional regulation of E2Fs may not be the only way by which RB can act as a tumour suppressor. A number of novel RB activities have emerged, and they serve to illustrate the multifunctional nature of this protein.

Cell cycle control through CDK inhibition

An E2F-independent mechanism for RB-induced cell cycle arrest has recently been uncovered. Ectopic expression of RB in an RB-deficient cell line induces a G1 arrest before E2F target genes become repressed⁵⁷. Thus, progression through G1 can be blocked, and CDKs can be inhibited, in an RB-dependent fashion, without altering the transcription of E2F gene targets. Two mechanisms, which are not mutually exclusive, have emerged to explain this observation (FIG. 3). In the first case, the CDK inhibitor p27 (also known as CDKN1B) is stabilized through the antagonism of its cognate F box protein, S phase kinase-associated protein 2 (SKP2). In this model, RB physically binds SKP2 through its unstructured RBC domain. This interaction prevents SKP2 from recognizing p27 and targeting it for degradation via the ubiquitin–proteasome system (FIG. 3a,b). Consequently, p27 is stabilized, causing CDK inhibition and cell cycle arrest⁵⁷. In the second mechanism, RB interacts simultaneously with SKP2 and the APC/C (anaphase-promoting complex; also known as the cyclosome)²². This interaction targets SKP2 for ubiquitylation and degradation, leading to the stabilization of p27, CDK inhibition and cell cycle arrest. In this model, again, RB acts as a scaffold, binding SKP2 at the RBC and the APC subunit CDC20 homologue 1 (CDH1) through the pocket (FIG. 3c). Although structural details of these interactions have not been elucidated, RB mutagenesis studies suggest that CDH1 binding involves the L-X-C-X-E-binding cleft in the pocket²². The RB–CDH1 interaction was further destabilized in an RB mutant that included mutations in both the L-X-C-X-E-binding cleft and the E2F-binding site in the pocket. An interesting possibility is that the same RB molecule cannot regulate E2F and APC–SKP2–p27 activities simultaneously. Studies suggest that APC and E2F binding to RB may be competitive, although the complexity of RB–E2F1 interactions described below suggests that this requires further investigation⁵⁸. Together, this mechanism of function and its apparent independence from E2F strongly suggest that RB is a multifunctional protein. Future structural studies will be needed to fully appreciate this mechanism and its regulation.

An E2F-independent paradigm of tumour suppression

To test the relevance of RB regulation of p27 *in vivo*, SKP2-deficient mice were crossed with animals heterozygous for *Rb*, and SKP2 loss was found to suppress cancer susceptibility of the *Rb*^{+/-} genotype⁵⁹. Surprisingly, it was discovered that inactivation of SKP2 in RB-deficient cells did not inhibit proliferation, but rather induced a p27-dependent apoptotic programme. Apoptosis leads to loss of the intermediate lobe of the pituitary, which is where tumours generally arise in *Rb*^{+/-} mice. It is noteworthy that in these mice, cells deficient in both RB and SKP2 exhibited deregulated E2F expression but not deregulated

proliferation. Again, this result suggests that RB-dependent regulation of non-E2F pathways is crucial. The existence of such pathways is more consistent with a multifunctional model of RB and is less supportive of an E2F-centric mechanism of function.

Heterochromatin and chromosome stability

RB has been shown to have an impact on genome stability through various distinct mechanisms.

E2F-dependent mechanisms

Overexpression of the E2F target gene mitotic arrest deficient 2 (*MAD2*) as a consequence of RB loss causes lagging chromosomes, in which chromosome arms become fused leading to double-strand DNA breaks or missegregation. These aberrations can result either from an overactive spindle assembly checkpoint⁶⁰ or from an inability to resolve merotelic microtubule attachments to sister chromatids⁶¹. Furthermore, deregulation of E2F transcription due to loss of RB function can lead to abnormally low nucleotide pools and replication stress⁶². This stress leads to double-strand DNA breaks and abnormal firing of replication origins and, ultimately, causes aneuploidy. Thus, loss of RB function has several negative effects on chromosome stability that are mediated by increased E2F activity, and the mechanistic involvement of RB in genome maintenance in these scenarios is probably best described by the classic mechanisms of E2F regulation described above. These paradigms of RB function exemplify the idea that regulation of E2F is paramount to RB, and that its diverse effects can ultimately be traced back to E2F control.

E2F-independent regulation of heterochromatin

RB and its family members have emerged as regulators of heterochromatin domains that surround the centromere and have a key role in chromosome structure and the attachment to spindle microtubules. Cells deficient for all RB family proteins display decondensed pericentromeric heterochromatin with reduced trimethylation of Lys20 on histone H4 (H4K20me3) as well as reduced DNA methylation⁶³. This defect leads to tangled chromosomes at metaphase, missegregation and aneuploidy. Many of these characteristics are observed in cells from a mouse strain bearing an RB mutation that impairs its L-X-C-X-E-binding cleft but not its E2F interactions, indicating that this chromosomal phenotype is caused by loss of just one aspect of RB function⁴⁰ (FIG. 4). The root cause of this phenotype remains uncertain. For example, loss of the histone tail modification H4K20me3 caused by deletion of the methyl transferases SUV420H1 (suppressor of variegation 4–20 homologue 1) and SUV420H2 similarly depletes this modification from pericentromeric regions but does not result in the chromosomal fusions seen in RB-deficient cells⁶⁴.

A series of recent reports have provided further insight into this aspect of RB function and narrowed down the possibilities for how this mechanism works. First, examination of the fruit fly orthologue of RB, called Rbf1, and comparison with fly mutations in components of the condensin II complex revealed similar phenotypes of chromatin decondensation, not only in mitotic chromosomes but also under growth arrest conditions²³. This report demonstrated a conserved interaction between RB and the condensin II complex in human

and fly cells. Furthermore, loss of RB function leads to defective loading of condensin II and cohesin, specifically at the centromeric region of mitotic chromosomes⁶⁵⁻⁶⁷. Phenotypically, this is identifiable in mitosis as defective chromosome congression, reduced cohesion and misaligned centromeres.

Beyond defects in cohesion and condensation of mitotic chromosomes, RB-deficient cells exhibit chromosome missegregation and aneuploidy. In addition to these mitosis-specific molecular defects, spontaneous double-strand breaks are seen in cells deficient for all RB family proteins⁶⁷. These defects reveal the means by which RB loss can influence genome stability independently of E2F transcriptional regulation. The effect of RB-mediated genome stability in cancer was investigated by crossing RB mutant mice that are viable but defective for L-X-C-X-E interactions with p53-deficient mice⁶⁶. In this way, compound mutant animals, and p53 knockout-only mice have similar G1 arrest defects. Compound mutant animals obtained from this cross succumbed to cancer significantly earlier than their p53-knockout controls. Furthermore, tumours were more metastatic in mice deficient in both RB and p53, and analysis of DNA copy number variation indicated that these mice had more alterations. Thus, maintenance of genome stability through the regulation of pericentromeric heterochromatin contributes to RB tumour suppressor activity.

Perspectives on RB regulation of chromosome stability

First, it should be noted that loss of RB does not universally cause chromosome instability⁶⁸. Studies of primary retinoblastoma tumours propagated in mice indicate that, in this tumour type, *Rb*-null mutations do not contribute to chromosome instability. However, given the relevance of the role of RB in cancer in a genomically unstable mouse model, understanding the mechanism of RB function in establishing heterochromatin at pericentromeric regions is vital. To this end, many fundamental questions remain (FIG. 3). Defects in condensin II and cohesin loading are central to the effect of RB on chromatin structure, but there are no data suggesting whether RB targets them to pericentromeric locations directly or regulates their loading by another means. Furthermore, phosphorylation by CDKs in late G1 typically inactivates RB by disrupting protein interactions², so it is difficult to envision how RB facilitates cohesion and condensation in M phase. Likewise, this aspect of RB function is dependent on interactions at the L-X-C-X-E-binding cleft, but the identity of the protein (or proteins) that it associates with is unknown. The condensin II subunit chromosome-associated protein D3 (CAPD3) has been proposed as a candidate²³, but there is little evidence that it directly contacts RB. RB could also bind other proteins in the condensin II ring or a separate bridging factor. Clearly, much work remains for us to understand this newly emerging aspect of RB function. However, its independence from E2F transcriptional regulation is well established and contributes to a growing body of literature that is revealing the multifunctional nature of RB.

Regulation of apoptosis

Largely through the study of E2F overexpression in cultured mammalian cells and deregulated E2Fs in RB-knockout mice, it was discovered that E2Fs could induce apoptosis⁶⁹. More recently, it has been found that apoptotic phenotypes in *Rb*-null mice are secondary to defective placental development⁷⁰, and hence are not exclusively caused by

overactive E2Fs. Almost simultaneously, new data have emerged describing a number of post-translational modification changes on E2F1 that allow it to respond to DNA damage and induce cell death³⁷. Specifically, E2F1 is phosphorylated by ataxia-telangiectasia mutated (ATM) and checkpoint kinase 2 (CHK2), two kinases that are activated by double-strand DNA breaks. E2F1 is also acetylated by p300/CBP-associated factor (PCAF; also known as KAT2B), an acetyltransferase that has been implicated in transcriptional control. In addition, several basic residues on E2F1 are also demethylated in response to DNA damage signalling. This level of regulation is unique to E2F1 as these modifications are not shared by other E2F family members. In general, the ability of RB to bind and inhibit E2F1-dependent transcription has been interpreted as a means to block apoptosis, and this is consistent with RB–E2F1 interactions ultimately mediating the effects of RB on cell viability. However, recent mechanistic advances in our understanding of RB and E2F1 in apoptosis now suggest multifunctional roles for these proteins.

DNA damage-induced alterations to RB–E2F1 function

A recent, transformative development in efforts to understand the role of RB in apoptotic regulation has been the discovery that it can have a pro-apoptotic role⁷¹. DNA damage induces the recruitment of PCAF to RB and E2F1, resulting in histone acetylation and transcription at specific pro-apoptotic promoters. To understand how RB can regulate both apoptosis and the cell cycle in response to different stimuli, it is important to consider how it regulates E2F1 in normal proliferating cells and how E2F1 control is altered in response to DNA damage.

The biochemical mechanism of E2F1 interactions with RB is different from that of other E2Fs. E2F1 was the E2F protein that was used to define the association between the RB pocket and the transactivation domain of E2F⁷². However, E2F1 has an additional interaction with RB that is unique among E2F family members²⁵ (FIG. 5a). The best evidence for the unique RB–E2F1 interaction is the demonstration that the adenoviral E1A protein can compete with and disrupt the common RB–E2F interaction but not the E2F1-specific interaction⁷³. Likewise, RB–E2F1 association persists when RB is phosphorylated⁷⁴. The structural determinants for the specific RB–E2F1 interaction have been mapped to the RBC²⁶. However, it is unclear to what extent the crystal structure of the RBC complex with the E2F1–DP1 marked box domains represents the E2F1-specific interaction^{12,74}.

The picture that has emerged from this differential regulation of E2F1 by RB is that CDK phosphorylation of RB releases most E2Fs from its control at the G1 to S phase boundary to stimulate transcription of cell cycle target genes, but at least some E2F1 remains bound to RB through the unique interaction of these proteins in S phase (FIG. 5b). This model explains a number of paradoxical reports that previously suggested RB–E2F1 interactions persist in S phase, even in the face of CDK phosphorylation^{75–77}. The unique RB–E2F1 interaction seems to negatively regulate transcription at pro-apoptotic promoters, such as TAp73, in reporter assays⁷⁴. In response to DNA damage, both RB and E2F1 undergo extensive changes in post-translational modifications³⁷. RB is dephosphorylated at CDK target sites but is phosphorylated by CHK2, as well as acetylated and methylated. E2F1 is

phosphorylated and acetylated as described above. The cumulative effect results in repression of the transcription of E2F-dependent cell cycle genes and the activation of pro-apoptotic genes (FIG. 5c). How these modifications lead to persistence of only the specific RB–E2F1 complex is unknown; however, two reports now demonstrate that a complex containing phosphorylated RB and E2F1 has pro-apoptotic activity and is found at the TAp73 promoter coincident with its activation^{71,78}.

Further experimentation to understand the molecular mechanism of RB–E2F1 transcriptional regulation will continue to be a challenging endeavour. It represents an area in which RB is again emerging as more than just an E2F regulator that is controlled by CDK phosphorylation. A deeper molecular understanding of RB–E2F1 interactions and regulation will undoubtedly clarify its role in apoptosis and the DNA-damage response and offer further evidence for the multifunctional nature of RB.

Reassessing the uses of RB function in cancer

Tumour specimens are often graded on proliferative indexes, and high levels of proliferating cells are generally correlated with poor outcomes. Recent advances in genomic analyses of tumours have established expression signatures that are indicative of deregulation of E2F activity^{79,80}. However, no markers are available that predict the ability of cancer cells to activate RB and restrict proliferation, or that predict its potential to stimulate apoptosis and eliminate tumours. Given the direct effect of genotoxic chemotherapeutics and novel kinase-inhibitor drugs for growth and survival pathways that affect RB and its downstream effectors, more rigorous classification of the functional state of RB in tumours will be beneficial for clinical outcomes. Furthermore, the key role of RB–E2F1 in regulating TAp73 expression in response to DNA damage offers tremendous potential for the development of therapeutics, as TAp73 expression has been shown to control sensitivity to genotoxic therapeutics⁸¹. A clearer structural picture of RB and E2F1 in their various interaction configurations will create the opportunity for therapeutic manipulation.

Conclusions and perspectives

Recent structural and functional data highlighted here are beginning to provide evidence that RB is a truly multifunctional protein. Current data also suggest that some novel functions of RB are potentially tumour suppressive. The RB–SKP2–p27 regulatory pathway is, seemingly, an E2F-independent proliferative control mechanism. Initial experiments demonstrate that CDH1 and E2F compete for RB association⁵⁸, and this type of biochemical insight defines RB as multifunctional. It is not clear whether CDK regulation through the RB–SKP2–p27 pathway is equivalent in importance with E2F transcriptional repression. The best available data suggest that both pathways affect the pituitary, but it is not yet possible to determine whether one pathway has a relatively more important role in a particular tissue. It is also possible that the RB–E2F and RB–SKP2–p27 pathways are crucial under distinct physiological circumstances that have yet to be determined.

A similar comparative discussion is possible for each of the different RB-dependent pathways described in this Review, and others that do not yet have an extensive structural understanding. Can RB engage in each one simultaneously as a higher order complex, or are

their interactions mutually exclusive, forcing cellular RB to be rationed among its different roles? Given the growing support for multifunctionality of RB, the next challenges in cell cycle and RB research will be to assign priority to these functions. Does RB rely more strongly on one function compared with others to act as a tumour suppressor? Only through the understanding and comparison of all functions in relation to one another will we appreciate the tumour suppressive role of RB in biochemical detail.

Acknowledgments

Research in the authors laboratories is supported by awards from the Canadian Institutes of Health Research (MOP89765 and MOP64253) and the Canadian Cancer Society Research Institute (2011-700720) to F.A.D, and the US National Institutes of Health (R01CA132685) and the American Cancer Society (RSG-12-131-01-CCG) to S.M.R. F.A.D is The Wolfe Senior Fellow in Tumour Suppressor Genes.

Glossary

Cyclin	A family of proteins that activate cyclin-dependent kinases and whose stability is cell-cycle regulated.
CDK	(Cyclin-dependent kinase). A family of kinases that are activated by cyclins.
E2F	(E2-binding factor). A family of cell-cycle regulated transcription factors.
Pocket	A region in RB-family proteins that was originally determined to bind to viral oncoproteins such as SV40 TAg
Differentiation-related polypeptide	(DP). The E2F dimerization partner.
Pocket proteins	RB-family proteins defined by their possession of the central 'pocket' domain
p27	An inhibitor of cyclin-dependent kinase activity.
F box protein	A protein containing the F box domain an approximately 50-amino acid motif that facilitates protein–protein interactions
S phase kinase-associated protein 2	(SKP2). An adaptor protein that recruits p27 to the SKP–cullin–F box E3 ligase complex.
APC/C	(Anaphase-promoting complex also known as the cyclosome). An E3 ubiquitin ligase.
Merotelic	When multiple microtubules emanating from opposite spindle poles, simultaneously bind to a single kinetochore
Aneuploidy	An abnormal number of chromosomes in a cell.
Centromere	A constricted region of a chromosome that interacts with kinetochores and is the attachment point for spindle microtubules.

Condensin II	A protein complex made up of seven protein subunits that create a ring structure to link and supercoil DNA strands.
Cohesin	A ring-structured protein complex similar to the condensins that creates cohesion between replicated homologous DNA strands and regulates their separation during cell division
Pericentromere	A repetitive chromosomal region adjacent to the centromere.

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Box 1**RB post-translational modifications**

Post-translational modifications have an important role in the regulation of RB function. With a few exceptions, RB phosphorylation (P) results in inactivation, transcriptional derepression and cell cycle progression³⁰. RB is phosphorylated by several different kinases, including cyclin-dependent kinases (CDKs) and checkpoint kinase 2 (CHK2)³⁷. Phosphorylation controls RB interactions with other proteins. This modification typically occurs outside structured domains (see the figure) and promotes conformational transitions from disordered to ordered RB structures that mask protein-binding surfaces^{12–14} (FIG. 1). Different kinases show preferences for particular phosphorylation sites, and discrete phosphorylation events induce specific structural changes. However, it remains uncertain whether, and in what context, differentially phosphorylated isoforms of RB exist in the cell.

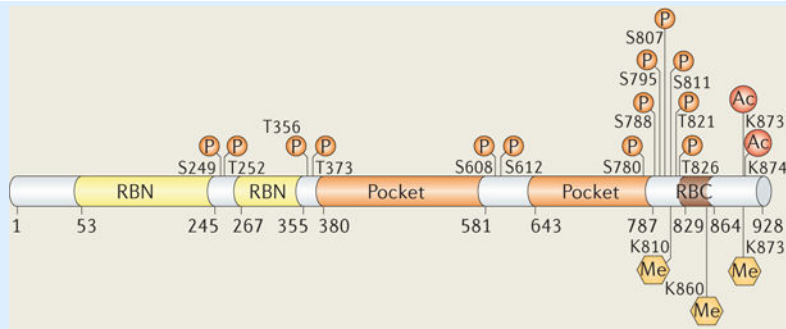
Acetylation (Ac) and methylation (Me) sites have been identified in disordered sequences towards the RB carboxy-terminal domain (RBC)³⁷. In contrast to phosphorylation, these modifications occur in response to signals, such as DNA damage and differentiation, which correlate with RB activation and repression of gene expression^{82–86}. Acetylation occurs on Lys873 and Lys874, which are located within the cyclin-docking sequence, and results in reduced phosphorylation, probably through kinase inhibition^{83,84}. Methylation on Lys873 and Lys810 by SET-domain methyltransferases similarly results in RB hypophosphorylation^{82,85}. SET and MYND domain-containing 2 (SMYD2) methylates Lys860, which results in the recruitment of the transcriptional repressor lethal(3)malignant brain tumour-like 1 (L3MBTL1)⁸⁶. The reader is referred to other reviews for more details on these and other emerging post-translational modifications on RB and their roles in the regulation of function^{33,37}.

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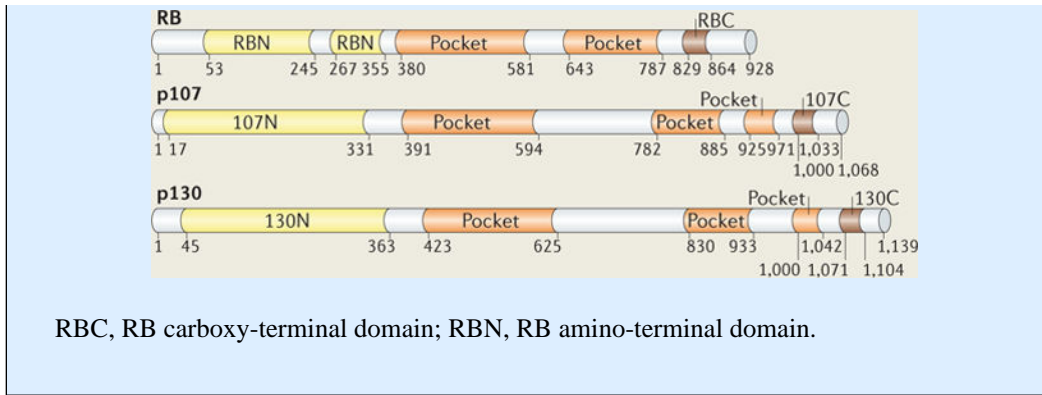


RBN, RB amino-terminal domain.

Box 2**The RB family of proteins**

RB, p107 and p130 are collectively known as the ‘pocket protein’ family. RB shares approximately 25% sequence identity with both homologues, whereas p107 and p130 share approximately 54% identity with each other. Structural data characterizing p107 and p130 are limited; however, sequence analysis suggests domains comparable to those in RB are present (see the figure). Both proteins contain predicted amino-terminal domains (107N and 130N), pocket domains and carboxy-terminal domains (107C and 130C), with analogous secondary and tertiary structural elements. Consistent with these parallel structural features, a number of common molecular functions have been identified^{87,88}. All three proteins negatively regulate the cell cycle, and for each protein this effect has been tied to its ability to associate with E2F transcription factor family members and influence E2F-mediated gene expression. The pocket domains of all homologues are predicted to contain L-X-C-X-E-binding clefts, which bind viral proteins and probably partially overlapping sets of cellular proteins. Pocket proteins are all inactivated by cyclin-dependent kinases (CDKs), and sequence analysis suggests several common structural effects of phosphorylation.

Genetic and cellular investigations have revealed various key functional differences between pocket proteins. RB knockout is embryonic lethal in mice, whereas knockout of p107 or p130 does not have a phenotype in a mixed genetic background^{89–91}. Examination of mice lacking different combinations of pocket protein genes suggests that p107 and p130 have an overlapping role in development that is distinct from that of RB⁹¹. Importantly, the tumour suppressor properties of the *RB* gene are significantly stronger than those of p107 and p130, and only RB mutations are commonly found in human cancer. Consistent with these genetic differences, pocket proteins have been observed to control distinct E2F target genes and arrest cells in different cell cycle phases⁴⁷. In a recent striking example, a genome-wide screen of gene repression in fibroblast cells revealed a unique role for RB in promoting senescence⁴². Further structural and biochemical analysis is needed to understand the molecular basis for these functional differences in pocket proteins, although distinct protein interactions have already been identified. For example, pocket proteins show preferences for different E2F family members, and p107 and p130 bind and inhibit CDKs, whereas RB exclusively forms a stable complex with protein phosphatase 1 (PP1)^{28,87,88}.



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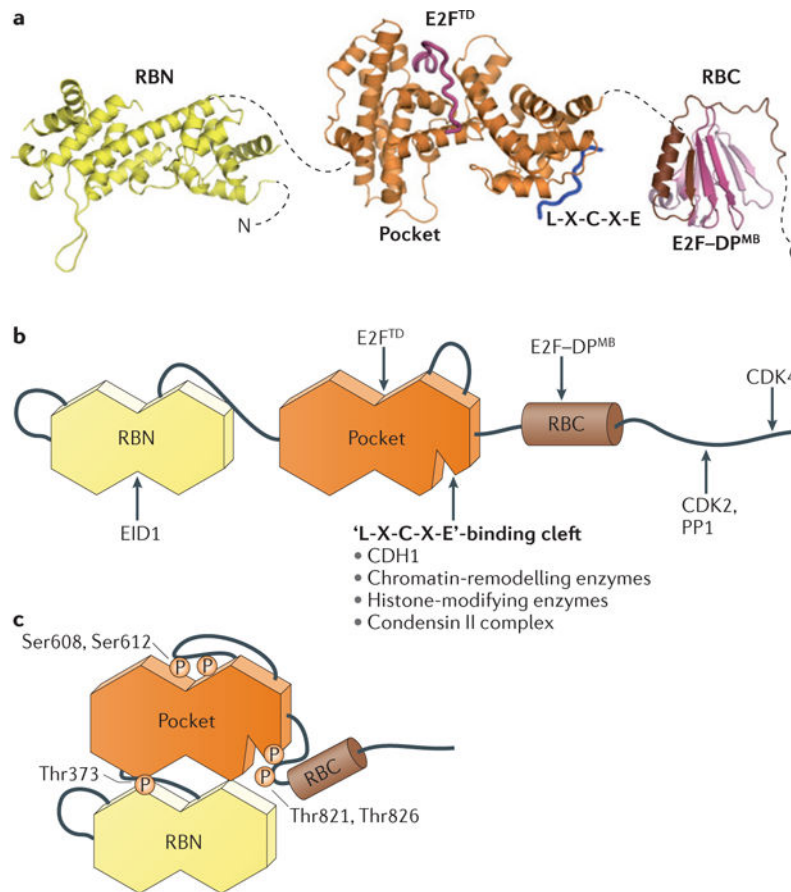


Figure 1. RB is a multidomain protein with several distinct protein-binding surfaces

a | Model of active and complexes with E2F and an 'L-X-C-X-E' peptide (Protein Data Bank (PDB) codes: 2QDJ, 1GUX, 1N4M and 2AZE). The dashed lines indicate flexible interdomain linkers. **b** | Schematic diagram of the domain structure and location of known binding sites for protein partners. **c** | Schematic structure of RB in its inactivated, phosphorylated conformation. Thr373 phosphorylation drives interdomain docking of the RB amino-terminal domain (RBN) and the 'pocket', whereas Ser608 and Ser612 and Thr821 and Thr826 phosphorylation induce binding of the pocket loop and the RB carboxy-terminal domain (RBC), respectively, to the pocket domain. These different conformational changes inhibit specific RB–protein interactions. CDH1, CDC20 homologue 1; CDK, cyclin-dependent kinase; DPMB, differentiation-related polypeptide marked box; E2F^{TD}, E2F transactivation domain; EID1, E1A-like inhibitor of differentiation 1; PP1, protein phosphatase 1.

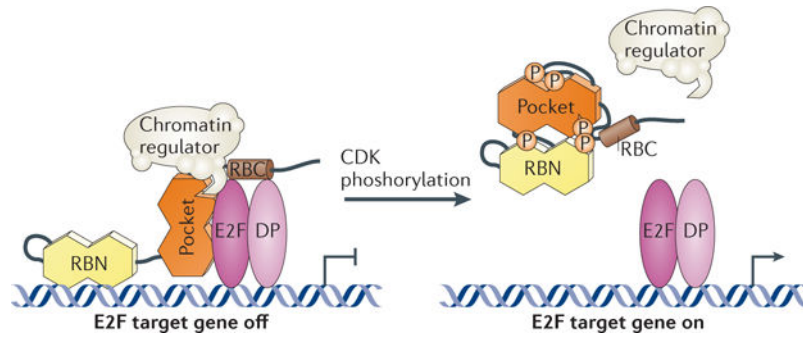


Figure 2. Regulation of E2Fs by RB

In the G1 phase of the cell cycle, RB is phosphorylated at low levels and associates with E2F transcription factors, which are dimeric proteins containing E2F and differentiation-related polypeptide (DP) subunits. RB also recruits enzymes that regulate chromatin structure to these complexes. Transcription of these genes is repressed until cyclin-dependent kinases (CDKs) phosphorylate (P) RB and prevent binding of E2Fs and chromatin regulators. E2F transcription factors then transcribe genes necessary for S phase and the cell cycle advances. RBC, RB carboxy-terminal domain; RBN, RB amino-terminal domain.

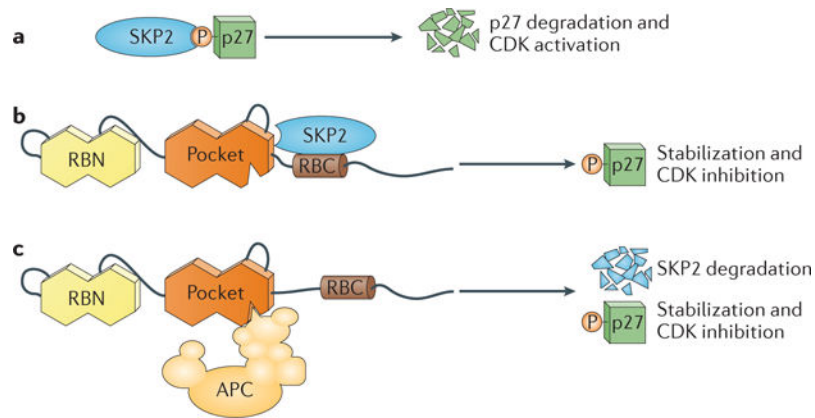


Figure 3. Transcription-independent regulation of cyclin-dependent kinases by RB

a | Schematic depiction of the F box protein S phase kinase-associated protein 2 (SKP2) recognizing phosphorylated p27. Binding results in ubiquitylation and degradation of p27 and the activation of cyclin-dependent kinases (CDKs). **b** | SKP2 is bound by the RB carboxy-terminal domain (RBC) and the ‘pocket’ domain, which competes with SKP2 for interaction with phosphorylated p27. RB thereby prevents p27 ubiquitylation, resulting in inhibition of CDK activity. **c** | The RBC region also binds SKP2 to recruit it to the APC/C (anaphase-promoting complex; also known as the cyclosome) E3 ubiquitin ligase complex. Ubiquitylated SKP2 (not shown) is targeted for degradation, leading to the increased expression of p27 and inhibition of CDKs. RBN, RB amino-terminal domain.

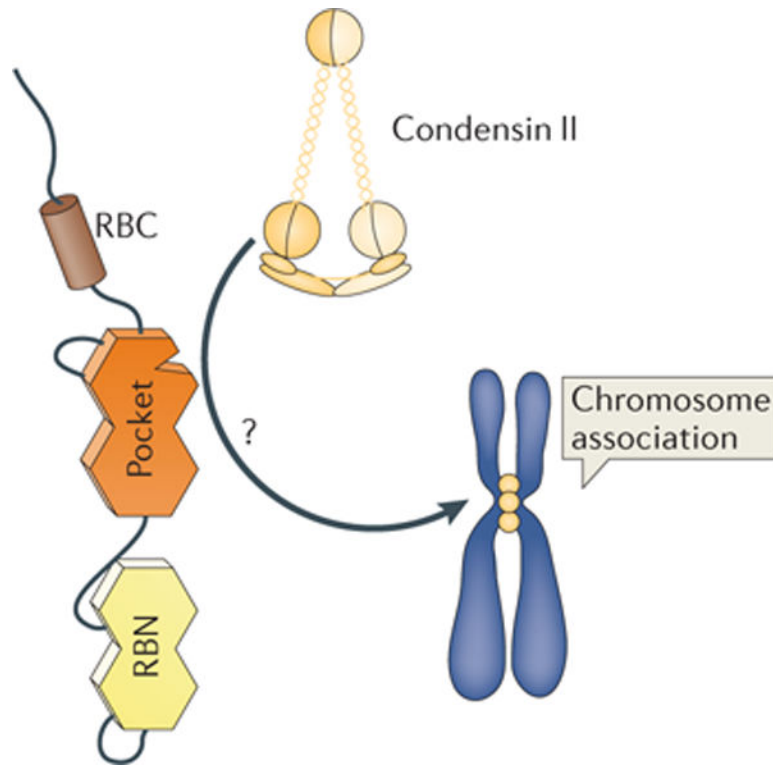


Figure 4. Regulation of pericentromeric heterochromatin by RB

The condensin II complex is enriched at pericentromeric and centromeric regions of mitotic chromosomes. Loading this complex onto chromatin is dependent on L-X-C-X-E-type interactions with RB. How this directs the condensin II complex to this genomic location is not known (indicated by a question mark), but it is suggested that additional proteins are involved. Similarly, the localization of cohesins to the centromere and pericentromere remains to be elucidated. Given the prominence of genome instability in mice deficient for L-X-C-X-E-type interactions, RB function in chromosome structure is key for the well-conserved L-X-C-X-E binding site. RBC, RB carboxy-terminal domain; RBN, RB amino-terminal domain.

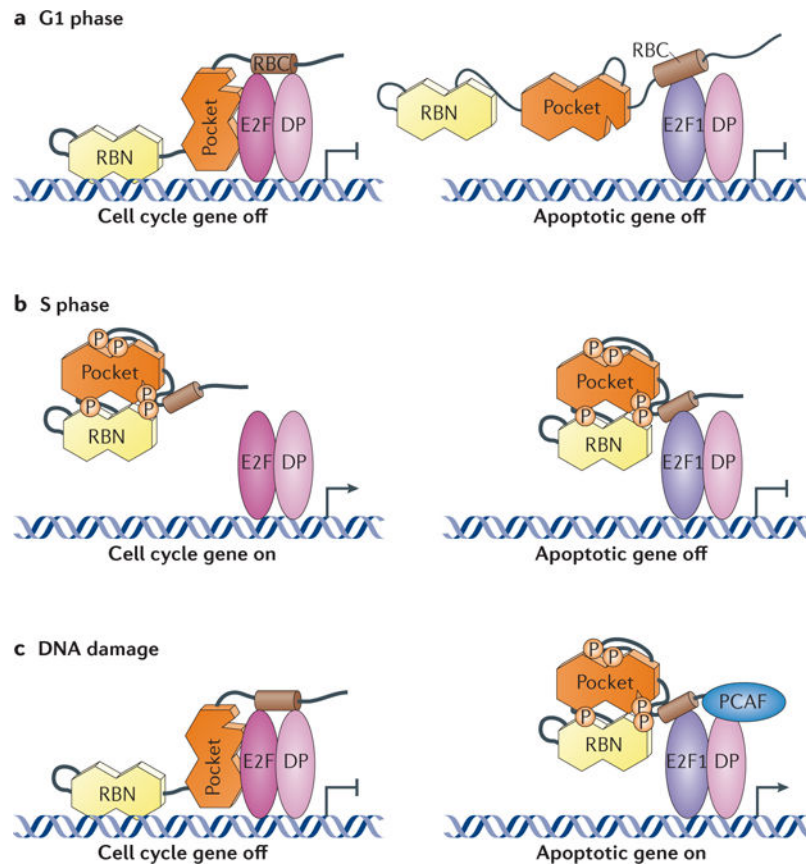


Figure 5. Differential regulation of E2F1 in apoptosis

a | RB associates with E2F transcription factors and differentiation-related polypeptide (DP) heterodimers in G1 to repress transcription of cell cycle genes. RB can also form a unique interaction with E2F1 involving sequences in the RB carboxy-terminal domain (RBC). **b** | In S phase, RB is phosphorylated (P) and unable to bind to E2Fs that are bound the promoters of cell cycle genes, allowing the cell cycle to advance. Phosphorylated RB can still interact with E2F1, and this complex can repress the expression of apoptotic target genes. **c** | In response to DNA damage, RB is dephosphorylated and regains the ability to repress E2F transcription at cell cycle promoters. Simultaneously, phosphorylated RB remains in contact with E2F1 transcription factors, and the recruitment of p300/CBP-associated factor (PCAF) through unknown signals leads to histone acetylation (not shown) and activation of pro-apoptotic target genes. RBN, RB amino-terminal domain.