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Review

# Retinoblastoma family of proteins and chromatin epigenetics: a repetitive story in a few LINEs

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## Abstract

The retinoblastoma (RB) protein family in mammals is composed of three members: pRB (or RB1), p107, and p130. Although these proteins do not directly bind DNA, they associate with the E2F family of transcription factors which function as DNA sequence-specific transcription factors. RB proteins alter gene transcription via direct interference with E2F functions, as well as recruitment of transcriptional repressors and corepressors that silence gene expression through DNA and histone modifications. E2F/RB complexes shape the chromatin landscape through recruitment to CpG-rich regions in the genome, thus making E2F/RB complexes function as local and global regulators of gene expression and chromatin dynamics. Recruitment of E2F/pRB to the long interspersed nuclear element (LINE1) promoter enhances the role that RB proteins play in genome-wide regulation of heterochromatin. LINE1 elements are dispersed throughout the genome and therefore recruitment of RB to the LINE1 promoter suggests that LINE1 could serve as the scaffold on which RB builds up heterochromatic regions that silence and shape large stretches of chromatin. We suggest that mutations in RB function might lead to global rearrangement of heterochromatic domains with concomitant retrotransposon reactivation and increased genomic instability. These novel roles for RB proteins open the epigenetic-based way for new pharmacological treatments of RB-associated diseases, namely inhibitors of histone and DNA methylation, as well as histone deacetylase inhibitors.

**Keywords:** chromatin; E2F; long interspersed nuclear element (LINE1 or L1); nucleosome; retinoblastoma protein family.

## Introduction

The mammalian retinoblastoma family of transcriptional repressor proteins is composed of three members: pRB (or RB1), p107, and p130 [for review, see (1)], discovered through characterization of the retinoblastoma gene product (pRB). *RB1* gene mutation and heredity were demonstrated to fit the two-hit hypothesis of cancer, with functions associated with cell cycle, oncogenesis, and cellular differentiation [for review, see (2–4)]. Furthermore, identification of the E2F family of proteins as RB partners in cell cycle regulation, coupled to ChIP on Chip analyses, have expanded our understanding of the global roles of RB proteins in cellular functions beyond cell cycle control. These novel functions are related to DNA and histone-mediated global epigenetic control of gene expression, genome integrity, chromatin function, cell development and differentiation, apoptosis, senescence, and embryogenesis (5–10). This review focuses on the role of RB proteins in the regulation of chromatin functions.

In normal cells, members of the E2F family of proteins bind to E2F binding sites on DNA in association with members of the DP family of proteins (E2F/DP heterodimers) and regulate gene expression of target genes (11). Also, RB family members target E2F proteins to form E2F/RB heterodimers (12, 13). Each of the RB proteins associate with E2F in a combinatorial manner, through regulation by cyclin-dependent kinases (CDKs) (14). Interaction of pRB with E2F at DNA promoters leads to transcriptional repression of genes associated with cell cycle progression, primarily genes involved in G1/S transition and DNA synthesis (12, 15). RB repressor functions are achieved via recruitment of repressor and corepressor proteins involved in local gene silencing, or direct interference with the E2F transactivation domain. Corepressor recruitment involves association of the E2F/RB complex with enzymes that alter the epigenetic code at the level of nucleosomal histones and DNA. These enzymes include histone deacetylase 1 (HDAC1) and DNA methyltransferase 1 (DNMT1) (16, 17); chromatin remodeling complexes, such as SWI/SNF (18), and histone methyltransferases (HMTs) (19). Interestingly, in quiescent cells arrested in G0, pRB is almost entirely absent, and p130, another RB family member is located at pRB target promoters (20). In addition, RB proteins contribute to the activity of cell cycle and chromatin regulators including DNMT1 (21), p27 (22), anaphase promoting complex, APC (23), and pRB itself (24). Hence, beyond the classical E2F/pRB pathway, RB proteins control cell cycle progression and genomic integrity through interaction with, and modulation of, effectors of multiple signaling pathways, as well as recruitment of chromatin modifiers of global genomic integrity (25, 26).

## Rb and oncogenesis

The characterization of *RB1* as a tumor suppressor was achieved through genetic studies of children affected by retinoblastoma [for review, see (27)]. pRB has also been associated with the onset of other tumors including osteosarcoma (2), soft tissue sarcomas (28), and DNA virus-induced cancers, such as cervical carcinoma and head and neck tumors (7, 29), and is believed to be dysregulated in the majority of human tumors (30). Genetic mouse models with combined deletion of different *Rb* family members have shown that RB proteins are involved in multiple cancers, and that their inactivation is required for cellular transformation (1, 31). We have shown that removal of *Rb* family leads to both increased acetylation of nucleosomal histones at LINE1 (L1) retroelement promoter coupled to decreased histone epigenetic silencing marks, which together lead to endogenous L1 reactivation (10). Thus, another mechanism for RB-induced tumorigenesis might involve L1 epigenetic reactivation and concomitant increased genomic instability. If intact RB functions are the required mechanism for L1 silencing, then the role of L1 in tumorigenesis could be attributed to reactivation following RB inactivation. However, this hypothesis has yet to be proven. The role of RB proteins in tumorigenesis is beyond the scope of this review, but multiple reviews on this topic are available (32–34).

## Retinoblastoma protein family and cell cycle control

The gene product pRB1/105 and related proteins, p107 and pRB2/p130, constitute a small family of phosphonuclear proteins, collectively referred to as pocket proteins [for review, see (35)]. The highly conserved pocket domain mediates interactions with cellular proteins that carry the consensus LXCXE motif. This interaction allows RB proteins to exert their biological function, and importantly to interact with viral oncoproteins, including human papillomavirus E7 proteins (36), adenovirus E1A protein, and large T antigens of Simian virus 40 (37–39). The pocket protein family is evolutionarily conserved from higher plants (with some variation among monocots and dicots) (40) to invertebrates and mammals (40, 41). This family is believed to function primarily as a regulator of the cell cycle (42), suppressor of cellular growth and inhibitor of cellular proliferation [for review, see (8, 35) and references herein]. The pocket domain has been implicated in mammalian development and differentiation, and mediates dynamic interactions between RB and other accessory proteins, including MyoD and HDAC1 in skeletal myoblasts (43), inhibition of cell fate determination in neuroendocrine lineages of the lung (44), control of differentiation in keratinocytes (45), cardiac myocyte differentiation (46), and multiple roles during embryogenesis [for reviews, see (1, 7, 8, 14, 35, 47)]. Members of the RB family of proteins exhibit differential expression patterns at various stages of the cell cycle in different tissues, as well as during embryogenesis (24, 48–50), indicating that the functional overlap among these proteins does not signify absolute redundancy (35). Of note is that pocket proteins play a key role in multiple processes in quiescent cells, whereas E2Fs are paramount for proper entry into cell cycle and successful transition of cells through G1, S, and G2 phases [for review, see (51)]. During G0 and early G1 the non-cycling cellular state is maintained through expression of 'repressor E2Fs' E2F4 and E2F5, which are bound to p130, hence repressing E2F-responsive genes required for DNA synthesis and replication, and mitotic processes (51). In differentiated or quiescent cells in G0, p130 in association with E2F-4/DP heterodimers is the most abundant E2F complex found and plays a role as cellular growth suppressor (35, 52, 53). In synchronous cultures of human cells derived from a glioblastoma multiforme tumor (T98G cells) progressing through early G1, as cells reenter the cell cycle, E2F-4 remains in association with p130. However, the promoter occupancy by E2F4/p130 greatly diminishes past mid-G1 (53). In mid- to late G1, p130 is replaced by p107, whereas in late G1 and S-phases, p130 is replaced by pRB/p105 at E2F target promoters (35, 54). The significance of these exchanges at E2F-regulated gene promoters is not fully understood. As cells progress into late G1, E2F-regulated promoters are occupied by 'activator E2Fs'; E2F1, E2F2, and E2F3 leading to transcriptional upregulation of cell cycle related genes at both G1/S and G2/M stages of the cell division cycle. This activation of gene expression is accompanied by increased acetylation of nucleosomal histones H3 and H4, characteristic markers of transcriptional activation (53).

The three pocket proteins of the RB protein family are post-transductionally regulated by cyclic events, such as serine/threonine residue phosphorylation. This mechanism relieves RB-imposed repression of E2F-regulated proliferation-promoting genes [for review, see (35, 55)]. Dephosphorylation leads to both RB stabilization through reduced ubiquitination and importin-mediated nuclear translocation (56), whereas acetylation at lysine residues on the C-terminus region is associated with cell cycle exit and cellular differentiation (57). Specifically, pRB nuclear phosphoprotein exerts its primary repressive function at early and mid stages of the G1 restriction point through transcriptional silencing of genes involved in G1- to S-phase transition, hence leading to cell cycle arrest at G1 (3, 58, 59). Nuclear hypophosphorylated RB proteins-induced arrest involves interaction with both activator and repressor E2F proteins bound to E2F-regulated genes, as well as recruitment of corepressor proteins with histone and DNA modifying properties (Figure 1) (4, 16, 19, 60, 61).

Cdks in complex with their cyclin catalytic partners form active holoenzymes that upon nuclear translocation modulate the RB protein repressor function by sequential phosphorylation during the G1 phase of the cell cycle (59, 62, 63). As a result, cells progress through the G1 restriction point and the S-phase of the cell cycle (59, 62). In total, 16 Cdk consensus sites spreading throughout the carboxy terminal domain, the amino terminal domain, and the pocket domain (seven, six, and three sites, respectively) have been identified within the human pRB amino acid sequence (64). Both

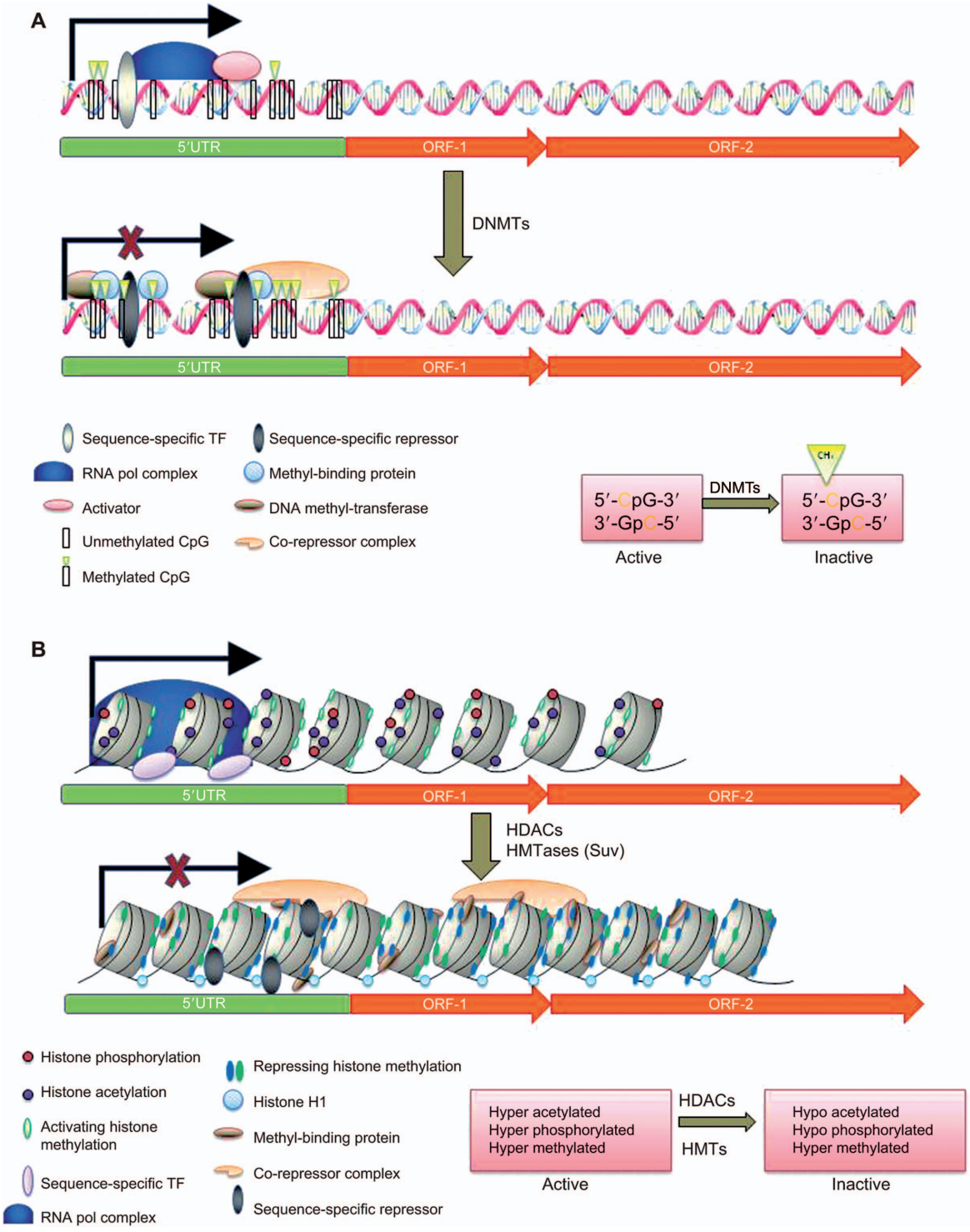


**Figure 1** Epigenetic regulation of gene expression.
Schematic representation of two epigenetic transcriptional control mechanisms: DNA methylation and nucleosomal histone modifications. (A) DNA methylation of CpG dinucleotides in CpG islands by DNA methyltransferases (DNMTs) leads to recruitment of transcription factors and accessory proteins involved in gene silencing and heterochromatin formation. The retinoblastoma transcription factor is able to repress transcription through direct recruitment of DNMT1. The L1 internal promoter (5′UTR) is subject to epigenetic control by E2F/RB and DNMTs (see text for details). (B) Epigenetic post-transcriptional modifications on nucleosomal histones lead to open or closed chromatin states responsible for activation or silencing of transcription, respectively. The retinoblastoma family of proteins interacts with both histone deacetylases (HDACs) and histone methyltransferases (HMTs) to silence chromatin leading to either facultative or constitutive heterochromatin formation. The nucleosomal proteins associated with the L1 internal promoter (5′UTR) are subject to epigenetic control by E2F/RB and HDACs (see text for details).

cyclins and their corresponding Cdks are activated during G1 and phosphorylate pRB in a stepwise manner. First, cyclin D1/cdk4 phosphorylates pRB specific amino acid residues, followed by cyclin E/cdk2 action in late G1-phase (59, 65–67). During S- and G2-phases, cyclin A forms complexes with cdk2 and cdk1 (also known as cdc2), whereas in mitosis cyclin B1 and B2 associate with Cdk1 and are responsible for maintaining pRB in the hyperphosphorylated state (67, 68).

Cdk activation of the mammalian cell cycle is tightly controlled by CDK inhibitor proteins (CKIs), including $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (cip/kip proteins) and inhibitor of kinase 4 (INK4) proteins ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$) (69). INK4 proteins block cyclin D/cdk4/6 activity by preventing the active holenzyme complex formation, thus leading to cell cycle arrest (70, 71), senescence, and tumor suppression [(72) and references therein]. However, evidence suggests that CKIs of the cip/kip family act as both negative and positive regulators of cyclin D/Cdk4/6 complex assembly (73). Furthermore, cyclin D/cdk4/6-mediated pRB hypophosphorylation might play an important role in promoting functional pRB/E2F4 complex formation in HaCaT keratinocyte cells (74). Thus, RB hypophosphorylation might be a step required for RB to selectively interact with specific members of the E2F family and/or other nuclear transcription factors, accessory proteins, or viral proteins, including adenovirus E1A oncoprotein (75). Cyclin E is usually present in cells in the form of an inactive complex predominantly bound to the $p27^{Kip1}$ Cdk inhibitor, and to a minor extent with $p21^{Cip1}$. Reversal of CKIs effect depends on several factors, including relative abundance of cyclins involved in inducible synthesis of cyclin D1 (76), *de novo* synthesis of cyclin E (77), and appearance of a 120-kDa active form that phosphorylates $p27^{Kip1}$ to relieve cyclin E/cdk2 from inhibition in a cell cycle-dependent manner (59, 78).

In all, the RB family of proteins exerts regulatory control of gene expression via modulation of transcription of genes regulated by E2F proteins. Likewise, RB proteins are subject to regulation via phosphorylation/dephosphorylation in a cell cycle-regulated manner. Unphosphorylated pRB is present at the quiescent G0 state, whereas hypophosphorylated pRB is found at early G0. pRB hyperphosphorylated forms can be detected at late G1 and throughout the remaining phases of the cell cycle, namely S, G2 and M. RB proteins bind to distinct members of the E2F family in a combinatorial manner to regulate expression of genes required for cell proliferation and cell cycle progression (35, 60, 79).

## Chromatin modifying proteins

In the past two decades, intensive efforts have focused on the dynamic interaction between DNA and histones, and its relation to gene expression, chromatin structure, and pathogenesis. New techniques have helped to unravel the complexity of chromatin structure and function. Chromatin is composed of a complex mixture of proteins, DNA, and RNA species. The basic particle of chromatin, the nucleosome, is made of a 147 nucleotide long, double-stranded DNA (dsDNA) wrapped around an octamer of H2A, H2B, H3, and H4 histone protein dimers [for review, see (80)]. Histones contain positively charged amino acidic residues in their protruding tails which are believed to interact with the negative charges of the dsDNA backbone, hence facilitating a strong interaction that maintains the DNA wrapped around the nucleosomal octamer. Nucleosomal histone tails are subject to a series of modifications including ubiquitination, sumoylation, phosphorylation, acetylation, carbonylation, and methylation (81, 82). These changes alter the strength of the interactions between histones and DNA, and lead to altered states of gene expression and genetic events including chromatin condensation, repair, and recombination [for review, see (83, 84)]. For instance, transfer of acetyl groups from acetyl-coenzyme A (acetyl-CoA) to positively charged amino acidic residues in histone tails (i.e., histone acetylation) by histone acetyl transferases is believed to decrease the interaction between DNA and the nucleosomal octamer. These in turn make the DNA more accessible to sequence-specific transcription factors, as well as chromatin modifying and transcription basal machinery, hence facilitating gene transcription (Figure 1) (85, 86). In contrast, HDACs are enzymes that catalyze the removal of acetyl groups from histone tails leading to a more compacted nucleosomal unit that is believed to silence gene transcription by making DNA less accessible to transcription factors [for review, see (87)]. Similarly, histone methylation plays a role in epigenetic control of gene expression and chromatin status. Histone residues that are targeted for acetylation can be targeted for methylation with opposite effects on gene expression; indeed, histone H3 lysine 9 acetylation leads to increased gene expression, whereas its methylation leads to gene silencing [for review, see (88, 89)]. Also, depending on the degree of methylation, this mark activates gene transcription or induces gene silencing and the formation of a more compacted chromatic structure (i.e., heterochromatin).

Mammalian HDACs are grouped into four classes: class I includes HDAC1, HDAC2, HDAC3, and HDAC8 which are homologous to yeast RPD3; class II is represented by HDAC4, HDAC5, HDAC7, and HDAC9 which are homologous to yeast HDA1; class IIa is represented by HDAC6 and HDAC10 which contain two catalytic domains; class IV which includes HDAC11; and lastly class III, also named sirtuins, which include SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7 and are homologs of the yeast *Saccharomyces cerevisiae* Sir2 protein [for review, see (90)]. These proteins do not bind directly to DNA and are usually recruited as part of corepressor complexes to DNA promoters and/or regulatory regions. The two major class I HDAC corepressor complexes, mainly Sin3 and NuRD, contain HDAC1 and HDAC2, and purify as megadalton corepressor complexes that play a key role in repression of gene transcription, and are required for embryo development (91, 92). Interestingly, these complexes remodel chromatin in an ATP-dependent manner, suggesting that nucleosomes are fluid particles subject to constant remodeling through histone tail modifications and nucleosome core displacement around the

DNA. Other protein complexes with nucleosome remodeling activity include SWI/SNF and RSC complexes (93). All of these multiprotein complexes contain subunits that although without apparent enzymatic activity can contribute to the integrity of complexes by acting as scaffolds, identifying and cooperating with specific substrates, and/or regulating corepressor function on chromatin substrates. These protein complexes associate with other corepressors and coregulators in combinatorial ways and serve multiple purposes in different cellular contexts. Owing to the complexity of these combinatorial networks, their functional roles and responses in multiple signaling pathways and developmental processes are not clearly understood.

Covalent modification of nucleosomal lysine residues include mono-, di-, or trimethylation of both histone H3 (H3K methylation) and histone H4 (H4K methylation). The best known marks for epigenetic silencing of chromatin include histone H3 trimethylation at lysine 9 (H3K9Me3) and histone H4 lysine 20 trimethylation (H4K20Me3) (9, 88, 89). The enzymes responsible for these covalent modifications are HMTs. Initial characterization of these proteins was performed in *Drosophila melanogaster* where three proteins, position effect variegation (PEV) suppressor SU(VAR)3–9, Polycomb group protein Enhancer of zeste, and trithorax group protein Trithorax (94, 95), were identified as SET domain-containing proteins [for review, see (9)]. In mammals, the homolog of Su(var)3–9 (SUV39H1) targets histone H3K9Me3 (81), and associates with HP1 protein, which is involved in gene silencing, DNA replication, conformation of constitutive heterochromatin, and nuclear architecture (96). Moreover, these proteins are essential for RB-mediated gene silencing and heterochromatin formation. Functionally, trimethylation modifications on histone tails are recognized by the bromo domain of HP1 protein and act as docking sites for heterochromatin nucleation in an HP1-dependent manner (97, 98). Histone H4K20Me3 reaction is mediated by SUV420H1 and SUV420H2 and Suv4-20h1 and Suv4-20h2 enzymes in humans and mice (99, 100). Interestingly, studies with mouse embryo fibroblasts indicate that the H4K20Me3 mark depends on the presence of Suv39h expression and the presence of trimethylated lysine 9 at histone H3. Similar to SUV39H1, Suv4–20 proteins interact with HP1 protein, suggesting a common role in transcriptional repression and heterochromatin formation (101, 102).

At the RNA level, small RNA inhibitory mechanisms also help mediate epigenetic silencing of gene expression and play a role in heterochromatin formation. Small RNA (RNA interference, RNAi, or post-transcriptional gene silencing) involves the processing of double-stranded RNA (dsRNA) precursors into small interfering RNAs (siRNAs) and microRNAs (miRNAs) by Dicer, an RNase III endoribonuclease [for review, see (103, 104)]. Next, proteins of the Argonaute family associate with dsRNA-bearing Dicer to form the RNA-induced silencing complex (RISC) which converts the dsRNA into single-stranded RNAs (ssRNAs). Through Watson-Crick base pairing this interaction identifies the target RNA for RNA degradation, or else can be used for establishment of heterochromatin sites following interaction with RNA polymerase II (105, 106), and subsequent recruitment of chromatin modifying complexes [for review, see (107–110)].

At the DNA level, cytosine methylation is the most widely studied mechanism for epigenetic silencing of chromatin and the establishment of stable heterochromatic states. The proteins involved in cytosine methylation belong to the DNA methyltransferase family (DNMTs) and include DNMT1, DNMT3a, DNMT3b, DNMT3L (which lacks the catalytic methylation domain), and DNMT2 with no known methyltransferase activity. Furthermore, DNMT1, DNMT3a, and DNMT3b transfer a methyl group to carbon 5 at cytosines followed by guanosines (CpG dinucleotides) [for review, see (9, 111, 112)]. Interestingly, these proteins are capable of binding DNA and are recruited to target regions through multiprotein repressor complexes. Important functions for DNMTs include (i) silencing of the X-chromosome (Lyon hypothesis) (113, 114); (ii) support of semiconservative maintenance of methylation patterns during DNA replication (maintenance methylation, via DNMT1) (115, 116); (iii) methylation of novel unmethylated CpG sequences (*de novo* methylation, via DNMT3a and DNMT3b) (117); and (iv) methylation of tRNA, with the participation of DNMT2 (118) [for review, see (9)]. Remarkably, a second type of DNA methylation, 5-hydroxymethylcytosine (5-hmC) has recently been identified. 5-hmC epigenetic marks result from the enzymatic conversion of 5-methylcytosine into 5-hydroxymethylcytosine by Tet-1 protein, which is a member of the ten-eleven translocation family of methylcytosine dioxygenases (119). Although a clear function has not been identified for 5-hmC, it is known to be present in relatively abundant levels in the mammalian brain, but not in other metabolically active, non-proliferating tissues. This finding suggests that it plays a role in controlling neuronal activity through epigenetic mechanisms that might include reduced binding affinity to proteins that recognize DNA methylcytosine, as seems to be the case for MeCP2 protein [(120) and references herein]. As with corepressor complexes, DNMTs play a key role in both development and disease. For instance, DNMT1 knockout mice are embryonically lethal and exhibit genome-wide demethylation of repetitive elements (121). Similarly, Dnmt3A$^{-/-}$ mice become runted and die close to 4 weeks after birth, whereas Dnmt3B$^{-/-}$ mice die *in utero* from multiple developmental defects (117). A third family member, Dnmt3L, seems to be required for methylation of repetitive sequences and maintenance of genomic imprinting (122).

It is important to note that multiple repressor/corepressor proteins act in concert to promote gene silencing and ensure specificity of epigenetic control of gene expression and chromatin function. This is best exemplified by the interaction of DNMT1 with HDAC1 (123), and the subsequent recruitment to E2F targets in an RB-dependent manner (16). Of note is that CpG dinucleotide-rich genomic regions, known as CpG islands because of their higher CpG content compared to other regions within the genome, have long been regarded as preferential sites of DNA methylation (Figure 1), and regions that mark functionally relevant epigenetic loci. More

recently, evolutionarily conserved regions of lower CpG dinucleotide content than CpG islands and located up to 2 kb away from promoters have been characterized as regions subject to methylation control and responsible for tissue-specific differentiation (124, 125). These regions are known as 'CpG island shores' and represent large stretches of DNA that could be targeted by methylated DNA-associated proteins and potentially providing a novel template for epigenetic control. Clearly, these sites could also be targets for epigenetic regulation by *RB1*, as discussed below.

## RB and epigenetics

RB proteins recruit corepressor protein complexes to E2F target promoters, whereas some of its binding partners, mainly E2F1 and E2F2, recruit histone acetylases to E2F target promoters, to promote a counterbalance to E2F-mediated gene activation. The ability of E2F/RB to bind non-canonical sequences rich in CpG sites (126) suggests a genome-wide involvement of RB proteins in chromatin structure and function. The transcriptional silencing mechanisms employed by RB proteins were initially explained through interference with E2F transcriptional activation by direct binding of RB to the E2F activation domain, and impairment of the ability of E2F to recruit general transcription factors, including the TATA-box binding protein (TBP) and TFIIH (127). An important step in understanding the mechanism(s) underlying RB family-mediated control of gene expression and chromatin structure came with the discovery of the direct association of HDAC1 and HDAC2 with pRB, and the ability of RB to recruit these enzymes to E2F-regulated promoters (17, 128). Furthermore, the proteins RbAp46 and RbAp48, which are members of most known mammalian corepressor complexes involving HDAC activity, including Sin3 and NuRD, were also shown to interact with pRB. This finding suggests that RB proteins associate with and recruit corepressor complexes to E2F target genes to exert gene silencing (18, 128, 129). Interestingly, the interaction between pRB and HDAC3 (a HDAC protein that opposite to HDAC1 and HDAC2 is mostly cytoplasmic), and RbAp48 (18), suggests that RB proteins could play alternate roles in cellular development and differentiation. The fact that RbAp proteins interact with RB has opened the way to understanding more complex mechanisms for the RB family involving chromatin modifications via protein complexes such as SMRT, N-CoR, and SWI/SNF (all of them containing HDAC3) (130, 131). Employing chromatin immunoprecipitation techniques, studies of the dynamic regulation of E2F target genes in quiescent and actively dividing cells showed evidence that HDAC1, HDAC2, and the Sin3A and Sin3B corepressor complex are bound to E2F target genes during quiescence. Also, HDAC1 and Sin3B are bound to B-myb, cdc2, E2F1, and cyclin A gene promoters in quiescent cells, but removed once cells re-enter the cell cycle (132). Interestingly, the most abundant promoter-associated complex involves p130/E2F4, suggesting that transient silencing of E2F targets through Sin3 corepressor in quiescent cells is mediated by p130 (132). Further interactions with histone modifying enzymes include the RB-mediated recruitment of HMTs, mostly members of the SUV family of proteins, to E2F targets with the concomitant formation of heterochromatic regions (99, 133). The interaction of RB with different members of the SET protein family, as well as with HP1 protein, probably facilitates both short- and long-term heterochromatin-mediated silencing of gene expression. Because histone methyltransferases play a role in centromere function and pericentromeric heterochromatin formation (134, 135), and the RB protein family is crucial for maintenance of pericentromeric chromatin (136), a concerted mechanism involving E2F/RB complexes and chromatin remodeling complexes (i.e., Sin3 and Sin3-like) can lead to formation and maintenance of facultative and constitutive heterochromatin. Studies with mouse cells that are triple deficient for the RB protein family (known as TKO cells) showed increased genomic instability in the form of aneuploid states and butterfly chromosomes, decreased DNA methylation levels, and activating histone marks [(9, 136) and references herein]. A striking feature was the presence of elongated telomeres, probably due to lack of proper assembly and condensation of higher order chromatin structures, also reinforcing the role of RB proteins in global chromatin structure and function. Some of these functions could be independent of E2F activity because cellular systems in which E2F has been displaced from promoters by a dominant negative E2F mutant carrying only the E2F DNA binding domain retained histone methylation profiles and telomere length was not affected (136). At a higher order chromatic structure, pRB associates with Condensin II complexes in an E2F-independent manner to regulate chromosome condensation during early mitosis (137). The question remains as to whether RB proteins integrate macromolecular complexes with other proteins in the absence of E2F, and the molecular signaling pathways which orchestrate the different arrays of corepressor complex recruitment to certain gene promoters, but not others. It seems that RB proteins act in combinatorial ways to modulate heterochromatin formation and cellular functions such as proliferation, development, differentiation, quiescence, and senescence (138–140). An important approach then will be to isolate and characterize the identity of macromolecular repressor complexes in which the RB proteins are present, as well as to correlate them with different stages of cellular differentiation. Stengel et al. (141) showed that antisera against pRB are a limiting factor for identifying pRB recruitment to promoters using ChIP approaches. Therefore, it would be of great value to characterize not only RB complexes but also the spatiotemporal and physiological conditions under which these interactions occur.

## LINEs, repetitive elements, and RB

Owing to their enrichment on CpG sequences, repetitive elements in the genome including retrotransposons have been shown to be hypermethylated in most normal somatic tissues. Human and mouse retrotransposons have bidirectional pro-

moters and are able to insert near or into genes, hence regulating gene expression through mutational and non-mutational events [for review, see (142, 143)]. Interestingly, mice contain a large number of active repetitive DNA sequences including retrotransposons of the L1Md family, with at least three highly active subfamilies $T_F$, $G_F$, and A (144–146) described along with mouse endogenous retroviruses. These repetitive elements account for the frequent epigenetic variation seen in mice (147, 148). Thus, repetitive sequences play an important role in control of gene expression, phenotypic variation, and epigenetic landscape in mice (149). In contrast, in humans only one family of L1 elements is present, and its expression is confined almost exclusively to reproductive tissues and endothelium [for review, see (150)], with only 80–100 still believed to remain retrotransposition-competent (151). DNA methylation, the most frequent mechanism for retroelement silencing is defective in tumor cells, leading to increased genomic instability, a hallmark of cancer. Interestingly, the lack of Rb family of proteins induces global hypomethylation and increased genomic instability (136). We have identified mouse and human retrotransposon LINE1 (L1) as RB targets and therefore a connection between RB, retrotransposons, and genomic instability is currently being actively explored (9, 10). Our studies comparing wild-type mouse embryo fibroblasts (MEFs) with MEFs deficient for the RB family of proteins (TKOs) showed that although impaired, the methylation levels of the L1 promoter did not suffer dramatic changes. It is probable that the DNA methylation machinery acts through redundant mechanisms that include not only RB-dependent recruitment of complexes involving DNA methylases but also direct recognition of methylated DNA by DNMTs, or other CpG-binding protein complexes, e.g., MeCP2 and MBD1. Remarkably, in a HeLa cellular model we recently identified crucial loci required for L1 activation. Out of seven CpG loci identified using MethPrimer software analysis, five proved to be important for L1 reactivation following siRNA degradation of human DNMTs, suggesting that not all CpGs are essential for silencing of L1 sequences (152). We found that human L1 CpGs are regulated at least in part through DNMT1 and DNMT3a and DNMT3b, suggesting that these proteins also regulate mouse L1 sequences even in the absence of RB (10, 152). If this is the case, then maintenance methylases could act, at least partially, independent of RB to maintain methylation of CpG loci on the L1 promoter. We do not know whether the same mechanisms apply to primary cells, and studies are underway to address this question. In a mouse model, we have observed a 10-fold difference in expression levels of L1 when comparing wild-type to TKO MEFs, indicating that key CpG loci are not properly methylated due to defective E2F/RB control of methylation (10). Although over the past 20 years our laboratory has focused primarily on protein complexes and their relation to repetitive element epigenetic control, the recruitment of RNA interference complexes to the L1 promoter, a process that might lead to heterochromatinization of this sequence, has yet to be explored. Small RNA mechanisms promise to reveal an exciting new mechanism for control of gene expression through recruitment of proteins involved in epigenetic modification of chromatin associated with silencing through heterochromatin formation. Remarkably, RB-interacting proteins of the polycomb family recruit non-coding RNAs to chromatin and induce gene silencing by promoting heterochromatin formation (153). To date, it is not clear whether this mechanism is involved in RB-mediated silencing. Furthermore, the potential linkages between non-coding RNAs, RB proteins, and repetitive elements remain largely unknown. Interestingly, in human cells the L1 bidirectional promoter leads to expression of miRNAs that could self-regulate L1 expression, thus interfering with retrotransposition events (154). Because mouse L1Md-A promoter is also bidirectional (155), it is probable that similar mechanisms participate in retroelement regulation of murine counterparts. It is important to note that the 5′UTR sequences in human and mouse retroelements differ in structural organization. Whereas human L1 5′UTR contains a unique sequence that is 903 bp in length (156), mouse L1 is made up of monomeric sequences 208 bp long that are organized in tandem to define their strength as a promoter (155). Whether bidirectional promoters are a recent evolutionary adaptation restricted to mice and humans, or whether these sequences also contribute to the establishment of global heterochromatin domains is unclear. Our laboratory is currently exploring the epigenetic control of L1 in the context of environmental stressors, namely benzo-a-pyrene and other persistent aromatic hydrocarbon carcinogens (152, 157–160). We have explored the response of L1 and the effects on RB and other accessory proteins on the L1 promoter. As mentioned above, RB regulates chromatin structure and function in regions rich in repetitive elements (pericentromeric regions) and also sequences in the genome that are rich in CpG islands (136, 161–163). This makes this group of proteins a candidate for global control of both transcription and facultative/constitutive heterochromatin formation. Because retrotransposons are repetitive elements that contain CpG islands in their promoters, we hypothesized that RB plays a role in the regulation of these elements via recruitment of HDACs and HMTs that generate the necessary epigenetic marks for heterochromatin formation and gene silencing (Figure 1). In the context of gene mutation, genotoxic exposure, and viral infection, defective RB function could lead to unwinding of the DNA and exposure of gene promoters to the basal transcriptional machinery due to loss of repressive heterochromatic marks in retrotransposon-rich regions, as well as gene-rich regions. If so, genomic instability could arise from increased retrotransposon gene expression and elevated retrotransposition rates that result in appearance of disease phenotypes. From a pharmacological perspective, the enzymatic functions afforded to multiple targets on the DNA by E2F and RB proteins provide a genome-wide opportunity for intervention in cancer. For instance, DNMTs, HDACs, and HMTs could become druggable targets in the treatment of human disease. Also, kinase inhibitors preventing RB hyperphosphorylation would enhance therapy outcomes by restoring the G1 restriction checkpoint controlled by the RB family of proteins. In all, RB control of repetitive and non-repetitive

sequences by multiple mechanisms (DNA and chromatin levels) play a major role in chromatin structure function. Many of these roles are not completely understood and therefore future studies are needed to characterize RB-associated macromolecular complexes, the spatiotemporal and functional modes of assembly, and the signaling pathways controlling recruitment of proteins. This work will open the door for better understanding of fundamental biological processes such as development and differentiation and the design of novel therapies for the treatment of multiple forms of human disease.

## Concluding remarks

The retinoblastoma family of proteins is essential at multiple levels: from cell, tissue, and organism development/differentiation and cell cycle control to chromatin structure and integrity. The multiple functions served by RB proteins in the organization and stabilization of heterochromatic regions inside and outside of pericentromeric domains implicate these proteins in maintenance of homeostatic balance and cellular integrity. Unveiling the macromolecular complexes that associate with RB proteins, the different signaling pathways that orchestrate their assembly, and the different target genes would help unravel the complexity of multiple interactions. This could provide multiple approaches for the use of RB proteins as pharmacological targets for treatment of cancer and other diseases.

The dysfunctionality of RB family members could contribute to pathogenesis through chromatin integrity-associated mechanisms including: (i) mediation of improper gene silencing (e.g., by contributing to aberrant DNA hypermethylation of tumor suppressors, including RB itself); (ii) increased genomic instability (e.g., through dysregulation of pericentromeric regions); and (iii) chromosomal segregation dysregulation (e.g., impaired interaction between RB and Condensin II) (137). Interestingly, in diseases such as acute myeloid leukemia (164) and ovarian tumorigenesis (165), the silencing marks H3K9me3 and H4K20me3 are associated with the establishment of heterochromatin domains around tumor suppressor genes. Thus, pharmacological intervention aimed at suppression of these epigenetic marks through DNMTs, HMTs, and HDAC inhibitors could lead to remodeling of chromatin and reactivation of mistakenly silenced tumor suppressor genes and provide further antitumor therapy alternatives.

In summary, RB proteins exhibit a new fascinating function, maintenance of heterochromatin function through interaction with multiple chromatin modifying enzymes. Dysfunctional RB proteins lead to both local and global changes in epigenetic changes that alter not only gene expression but also chromatin structure, genomic instability, and chromosomal rigidity and segregation. Thus, targeting of RB-associated proteins through pharmacological agents such as HDAC inhibitors, HMT inhibitors, and DNMT inhibitors make RB a desirable target for novel antitumor therapies.

## Outlook

The field of epigenetics, boosted by 'omics' technologies has provided insight into the dynamic role that histone and non-histone proteins play in regulation of cellular events ranging from pluripotency to differentiation and disease. We envision the nuclear compartment as a dynamic unit that contains individual processing units that have evolved to make the genetic and molecular response to environmental cues as accurate as possible, and therefore chromatin structure and function is a mere reflection of this order. In other words, the enrichment of L1 and other repetitive sequences at given regions within the genome, and the subnuclear compartmentalization of proteins and genetic material could entail highly relevant correlations that have not yet been addressed. These interactions could reveal a great deal of information about chromatin structure/function and the functional role that repetitive elements play on genomic integrity and cellular function. For instance, it is tempting to speculate that L1 elements, which rather than being confined to chromosomal domains are spread throughout the genome, have been selected through evolution to serve as scaffolds used by RB proteins. In so doing, the interaction could orchestrate nucleation of heterochromatin domains and maintenance of chromosomal condensation and rigidity during mitosis. In addition, understanding the role that cell signaling plays on reordering of protein complexes will prove paramount for the dissection of molecular pathways associated with both gene silencing and expression, as well as genome-wide changes in chromatin architecture, including regulation of euchromatic and heterochromatic domains (both facultative and constitutive). Targeting these signaling pathways at the nuclear level will help address the puzzle of differentiation and disease, and lead to the development of more accurate and targeted epigenetic drugs.

## Highlights

- Recent evidence shows that RB proteins are involved in multiple cellular processes associated with cellular proliferation, senescence, apoptosis, and maintenance of heterochromatin structure.
- RB proteins play a key role in maintenance of global architecture. At the histone level, RB proteins help stabilize the nucleosomal epigenetic marks H3K9me3 and H4K20me3 in pericentromeric regions, rich in short repetitive sequences, as well as in retroelements spread throughout the genome. This role also correlates with the recruitment of HMTs in a RB-mediated manner.
- E2F/RB complexes localize not only to E2F DNA consensus sequences but also to CpG-rich regions thus conferring a genome-wide role for these heterodimers in control of gene function (local) and chromatin integrity (global).
- LINEs and other repetitive sequences might not only be parasitic elements but might rather have evolved to function as the scaffold on which E2F/RB and other chromatin

modifying transcription factors and structural proteins rely for proper organization of different heterochromatic domains and for maintenance of chromatin integrity and chromosomal compaction.

- Important issues to be addressed in the future include (i) the role of siRNA and signaling pathways in macromolecular protein complex assembly and genome-wide control of heterochromatin, (ii) the role of RB in L1-mediated genomic instability, and (iii) the contribution of repetitive elements to global composition of facultative heterochromatin as well as gene expression (both aberrant and during morphogenesis).
- Finally, we believe that the L1 promoter is regulated by several protein complexes that maintain it within facultative heterochromatin, suggesting that these elements are readily reactivated following genotoxic injury. Identifying those proteins and the mechanisms that control their recruitment and disassembly from LINE regulatory regions will shed light into the fundamental processes that lead to disease as well as regulation of development and differentiation.
- From our perspective, the most important question to address is: what biological functions L1 elements play within the genome and what is the cellular context required for such functions? Why does L1 become reactivated following genotoxic exposure? Do the proteins encoded within L1 participate in the stress response? And if so, how?

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