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Tumour suppressor retinoblastoma protein Rb: A transcriptional regulator

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Abstract

Rb was the first tumour suppressor identified through human genetic studies. The most significant achievement after almost twenty years since its cloning is the revelation that Rb possesses functions of a transcription regulator. Rb serves as a transducer between the cell cycle machinery and promoter-specific transcription factors. In this capacity, Rb is best known as a repressor of the E2F/DP family of transcription factors, which regulate expression of genes involved in cell proliferation and survival. An equally important aspect of Rb as a transcription regulator is that Rb also activates certain differentiation transcription factors to promote cellular differentiation. The molecular mechanisms behind the repressive effects of Rb on E2Fs have come to light in significant details, while those relating to Rb activation of differentiation transcription factors are much less understood. Finally, it has become clear that there are other aspects to Rb function that are not immediately related to transcription regulation.

Keywords: Retinoblastoma protein Rb; Tumour suppressor; Cell cycle; Differentiation; Transcription; E2F; Chromatin; p27Kip1; Skp2; Cell cycle; Oncoprotein; Protein degradation; Partial penetrance

1. Introduction

Retinoblastoma syndrome is mostly a childhood malignancy with family history. Human genetic studies of this disease and the two-hit hypothesis of the tumour suppressor gene concept [1] led to the cloning of the gene for the retinoblastoma protein Rb in 1986 [2], which encodes a nuclear protein of 928 amino acids. The tumour suppressor nature of the Rb gene and the two-hit hypothesis was subsequently tested and confirmed experimentally in the mouse. Mice engineered to inherit a null allele of Rb gene invariably develop tumours in the pituitary and thyroid glands, and tumour cells invariably lose the wild type allele of the Rb gene [3–5].

Later in life, retinoblastoma patients also develop tumours in other tissues. Sporadic somatic mutations in the Rb gene have also been identified in various cancers, indicating that the tumour suppressor role of Rb is not restricted to the retina [6]. Furthermore, Rb protein can be inactivated by phosphorylation by cyclin-dependent kinases (in particular cyclin D/Cdk4). Overexpression of cyclin D1, activating mutations in Cdk4, and inactivating mutations in the cyclin D/Cdk4 inhibitor p16Ink4a are frequent events in various human cancers. Since most, if not all, human cancers have one or more of these events, it has been proposed that disruption of Rb function is a general feature of cancer cells [7].

An important component in Rb studies was the identification of the two Rb related proteins p107 and p130, which are more closely related to each other than either one is to Rb [8,9]. These three proteins are all targeted by viral onco-proteins for cell transformation. The shared sequences for onco-protein binding are called

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the "pocket" (hence these three proteins are often called pocket proteins). Interestingly, mutations in p107 and p130 are extremely rare in human cancers. It is currently believed that p107 and p130 share tumour suppression activities with Rb since combined knockout of p107 or p130 is required for Rb deficient retinal progenitor cells to develop retinoblastomas and for Rb heterozygous mice to develop a wide spectrum of tumours [10–13]. The closer resemblance between p107 and p130 may provide adequate functional redundancy to compensate for the loss of either one of them. Almost all Rb studies have included p107 and p130; this review however will focus on Rb, while a more detailed discussion of p107 and p130 can be found elsewhere [8,9,14,15].

Assay protocols that express wild type Rb in Rb mutant tumour cells have been used to study Rb function [16,17]. Results from these assays suggest that Rb can inhibit cell cycle progression from G1 to S [17,18]; can induce a senescence-like phenotype [19,20], and can activate certain bone differentiation markers in osteosarcoma cells [21,22]. These activities of Rb do not depend on high levels of expression and are abolished by Rb phosphorylation or mutations isolated from retinoblastoma samples [18,23,24]. Conversely, Rb knockout mouse embryonic fibroblasts (MEFs) enter S phase faster than Rb wild type MEFs after serum re-stimulation [25], and Rb knockout MEFs are compromised in their ability to arrest G1–S progression in response to DNA damage [26,27].

Mouse genetic studies also revealed that Rb functions are essential for embryonic development. Rb null embryos die at 13.5 days of gestation with prominent defects in the central nervous system and hematopoietic system [3–5]. Deregulated proliferation and cell death were observed in these tissues as a result of both cell autonomous and non-cell autonomous effects of Rb inactivation [28]. These phenotypes, reviewed in [29,30], establish an increasingly recognised connection between regulatory mechanisms for animal development and for tumourigenesis.

2. Regulation of E2F transcription factors by Rb

Rb does not posses sequence-specific DNA binding activity but can interact with many sequence-specific transcription factors, the best understood of which is the E2F transcription factor family [31–35]. Rb serves as a transducer between the cell cycle machinery and E2F. In cell cycle stages when cyclin-dependent kinases are not active (early G1 phase, quiescence, or senescence), Rb is hypo-phosphorylated and interacts with E2F. When cyclin-dependent kinases are activated during G1–S transition, Rb becomes hyper-phosphorylated and loses its ability to interact with E2F. Mutations of Rb identified in retinoblastoma samples also disrupt the ability of Rb to interact and repress E2F, suggesting that repression of E2F is relevant to Rb tumour suppression activity.

E2F family of transcription factors consists of E2F and DP sub-groups. To date, there are eight E2Fs and two DPs [34–37]. E2F1, 2, and 3 are traditionally believed to be "activator E2Fs" governed by Rb while E2F4 and 5 "repressor E2Fs" governed by p107/p130, although this division is not absolute [38–40]. An interesting feature of the extended E2F family is that newer members (E2F6, 7, and 8) do not contain transactivation and Rb binding domains. Another new recognition is that E2F proteins regulate a broad spectrum of genes involved in cell cycle regulation, DNA damage response, apoptosis, differentiation, development, as well as many genes of currently unknown function [41–45]. Similarly, genome wide studies with Rb family proteins have revealed roles well beyond the regulation of E2F and the cell cycle [46–48].

With so many members in the E2F family, one would expect that each might regulate a specific subset of E2F target genes. Indeed, inactivation of different E2F family members in mice yields very different phenotypes [36]. This specificity apparently is not due to preferences of different E2F members for any particular variations in the E2F DNA binding consensus sequences. Rather, other promoter-specific transcription factors can interact and synergise with specific members of E2F family to regulate specific promoters [49–52]. This mechanism for specific E2F members may also be applicable to the Rb family members. The formation of a specific Smad3–p107–E2F4/5 complex and the adjacent Smad and E2F binding sites in the c-myc promoter form the molecular basis for the rapid inhibition of c-myc expression by p107 in response to TGFβ treatment that can not be compensated by Rb or p130 [53].

The functional relevance of Rb repression of E2F in tumourigenesis has been tested in the mouse by combining Rb+/− with various E2F knockout mutations. Knockout of E2F1, E2F3, or E2F4 delayed pituitary and thyroid tumourigenesis in Rb+/− mice, providing genetic evidence for the role of Rb-mediated E2F repression in Rb-mediated tumour suppression [38,54,55].

3. Mechanisms of Rb-mediated repression of E2F

3.1. Physical interference with E2F ability to interact with the basic transcription machinery

Since the transactivation domain and Rb binding domain physically overlap at E2F C-termini [56,57], the simplest mechanism for Rb-mediated repression is where Rb binding physically interferes with the ability of the transactivation domain to communicate with the basic transcription machinery. This scenario has

indeed been experimentally demonstrated with purified proteins and naked DNA promoter sequences [58]. This simple mechanism however is insufficient to account for several earlier findings. Rb can reduce the E2F reporter activity to levels below that in the absence of E2F [59]. When Rb was artificially tethered to promoter DNA with a fused GAL4 DNA binding domain [60,61] or a fused E2F DNA binding domain [62], it could act as a repressor on the basic promoters.

3.2. Covalent modifications of chromatin histones in Rb-mediated repression

The basic unit of chromatin is the nucleosome, where approximately 147 bp of DNA wraps two rounds on a protein core consisting of two molecules of each histone: H2A, H2B, H3 and H4. The nucleosome structure possesses significant influences on processes that require direct contact with DNA such as transcription. Access to DNA can be regulated by post-translational modification of the extruding histone amino-termini with acetylation, phosphorylation, and methylation. Among them, histone acetylation and methylation have been well studied in the past few years, which coincide with rapid progress in our understanding of the mechanisms underlying Rb-mediated repression of transcription. Acetylation has been identified on lysines 9, 14, 18 and 23 of H3, and lysines 5, 8, 12, and 16 of H4, and correlates with active transcription. Methylation of lysines 9 and 27 of histone H3 and lysine 20 of histone H4 are indicative of transcriptionally inactive chromatin, while methylation of lysines 4, 36 and 79 are correlated with transcriptionally active chromatin. Each lysine can be mono-, di, or tri-methylated. Arginine residues at 2, 17, and 26 of H3 and 3 of H4 can also be methylated, which often correlates with activation by certain nuclear receptors [63]. A connection between these histone modifications and cell signalling pathways seems to be provided by histone H3 phosphorylation at serine 10. This residue can be phosphorylated by the kinase Rsk-2 in response to EFG signalling [64] or by IκB kinase IKKα in response to cytokine [65,66]. Phosphorylation of serine 10 of histone H3 can promote acetylation of lysine 14 [67–69]. On the other hand, methylation of lysine 9 can interfere with serine 10 phosphorylation [70]. Various combinations of these modifications may constitute codes that dictate chromatin status [71,72].

Acetylation of lysine ε-amino group neutralises its positive charge, which can decrease the affinity of histones for negatively charged DNA to open up access to the DNA. Since their first identification, a large number of histone acetyltransferases (HATs [73]) and histone deacetylases (HDACs, [74]) have been identified [75,76]. Many transcription factors and co-activators have intrinsic HAT activity, and, conversely, many co-repressors have intrinsic HDAC activity. E2F has been found to associate with various HATs including p300/CBP, P/CAF, and Tip60 [39,77–80]. Like many other transcription factors, E2F itself can be acetylated by p300/CBP and PCAF, which can increase its DNA binding and transactivation activity as well as protein stability [81,82].

Rb can interact with HDAC1, 2, and 3 and recruit them to the promoters of E2F target genes to affect repression [83–86]. Overexpression of these HDACs can enhance Rb ability to repress E2F target genes; and the HDAC inhibitor trichostatin A (TSA) can abolish Rb repression on some, but not all, E2F target promoters. HDAC1 and 2 contain the consensus Rb binding motif LXCXE but HDAC3 does not. It has been shown that Rb–HDAC interaction is mediated by RBP1, an Rb interacting protein with two separable domains for interaction with HDACs and an unidentified repression activity that is not sensitive to TSA [87]. Recruitment of HDACs to E2F can also result in deacetylation of E2F as another mechanism for repression [81].

Histone methylation is considered a long-term mechanism of transcription regulation since methylation is chemically stable [88]. For example, combination of H3-K9 di-methylation, H3-K27 tri-methylation and H4-K20 mono-methylation has been suggested to be the code for the generation of the inactive X chromosome. A combination of H3-K9 tri-methylation, H3-K27 mono-methylation, and H4-K20 tri-methylation may be the code for the formation of the constitutive pericentric heterochromatin. A number of histone lysine methyltransferases have been identified based on the conserved SET domain structure [63]. Interestingly, the most recently identified H3-K4 methyltransferase SMYD3 is found overexpressed in colorectal cancers and hepatocellular carcinomas [89], adding evidence to the increasingly clearer theme that histone modification plays an important role in tumourigenesis [90].

Clues to how histone methylation facilitates repression came from the fact that acetylation and methylation of H3-K9 are mutually exclusive. Therefore, deacetylation of H3-K9 by HDAC, which leads to repression, also makes it possible for H3-K9 to be methylated by the methyltransferase Suv39H [70]. Since methylation is chemically more stable than acetylation, H3-K9 methylation may serve to "lock in" the deacetylated status of H3-K9 for long-term repression. Another important feature of histone lysine methylation is the binding of heterochromatin protein 1 (HP1) to methylated H3-K9 [91,92]. HP1 also interacts with a number of chromatin modifying activities (including methyltransferases) and therefore could recruit them onto the methylated chromatin in a step-wise spreading process to generate heterochromatin. How methylation at certain lysine and arginine residues can activate transcription remains unknown. How methyltransferases

are first recruited to DNA is now beginning to be unraveled.

Since methylation of H3-K9 by Suv39H and the subsequent binding of methylated H3-K9 by HP1 has been associated with heterochromatin, it was quite a surprise when Rb was found to interact with Suv39H and recruit it to the cyclin E promoter to facilitate H3-K9 methylation and HP1 binding as a mechanism of repressing the cyclin E promoter [93], which is regulated in a dynamic process in every cell cycle [94]. Histone methylation has not been viewed as a dynamic process partly due to the elusive nature of demethylases. Recently however, a lysine-specific demethylase (LSD) has been identified [95]. Although LSD targets H3-K4 to suppress activation, it suggests the possibility that more demethylases will soon be identified that could remove repressive methyl modifications to reverse repression. The histone methylation processes may not be as permanent as previously thought [88].

Nevertheless, the connection between Rb and histone methyltransferases has indeed been linked to long-term gene silencing. Narita and co-workers recently reported that various means of inducing senescence in certain human primary fibroblasts generates a distinctive heterochromatin-like structure called SAHF (senescence-associated heterochromatic foci). In SAHFs, gene expression was stably silenced such that E2F target genes could not be activated by enforced expression of E2F1. These E2F target promoters are methylated at H3-K9 and are associated with HP1. In this scenario, Rb was detected on promoters of cyclin A and PCNA and is required for the generation of SAHF. This study [96] thus demonstrates a connection between Rb and long-term repression consistent with Rb recruiting the methyltransferase Suv39H and HP1 to E2F target promoters [93]. To carry this theme further, a recent report demonstrated that Rb, together with p107 and p130, play a more general role in the generation and maintenance of heterochromatin through direct interaction with another histone methyltransferase Suv4-20H [97], which targets H4-K20 for tri-methylation [98]. This process however does not seem to involve E2F. It is not clear whether the pocket proteins are recruited to chromatin by the Suv4-20H or vice versa.

3.3. Polycomb group protein complexes and Rb-mediated repression

Polycomb group proteins (PcG) belong to large, multi-subunit protein complexes originally identified as important regulators of Homeotic genes in *Drosophila* embryo [99,100]. They provide long-term and heritable gene silencing to sets of genes. There are two distinct types of PcG complexes: polycomb repressive complex 2 (PRC2, also called the initiation complexes) and polycomb repressive complex 1 (PRC1, also called the maintenance complexes). Biochemical purification of PRC2 revealed the presence of a SET domain-containing protein E(z)/EZH, which can methylate H3-K27 [101–104]. Tri-methylated H3-K27 forms a binding site for the polycomb protein Pc/HPC in PRC1, suggesting a mechanism for PRC2 to recruit PRC1 to the chromatin. PRC1 complexes can achieve repression by physical hindrance against access to DNA, by attracting HDACs and methyltransferases, by blocking activating methylation of H3-K4, and by blocking access of chromatin remodeling complexes (discussed below). The mechanism that is more important for long-term silencing is not clear but they are not mutually exclusive.

The connection between PcG proteins and cancer was provided by the isolation of a PRC1 complex subunit Bmi1 as an oncogene that could cooperate with myc in mouse lymphomagenesis [105,106]. It was later determined that Bmi1 is a potent repressor of the Ink4a/Arf locus [107], which encodes both p16Ink4a and p14Arf. p16Ink4a inhibits cyclin D-dependent kinase activity to activate Rb, while p14Arf inhibits MDM2 activity to activate p53. Bmi1, as well as the PRC2 subunits EZH2 and SU(Z)12 are often overexpressed in various human cancers [108].

The connection between Rb and PcG proteins was first made in search of proteins that are involved in Rb-mediated HDAC-independent repression. A protein called CtIP was isolated on the basis of its interaction with p130 and Rb [109]. The N-terminus of CtIP mediates the interaction with Rb/p130 while the conserved PLDLS sequence interacts with the co-repressor CtBP. One of the potential mechanisms for CtBP repression function was hinted by its interaction with the polycomb protein HPC2 in PRC2 [110]. Dahiya and co-workers then showed that Rb can form a complex with CtBP and HPC2, which is required for Rb repression of cyclin A and Cdc2 promoters [111]. Rb and HPC2 also cooperate in arresting HPC2 deficient cells. Whether cellular Rb and HPC2 also interact however was not clearly established [112]. Interestingly, E2F6, which does not interact with Rb due to the lack of the C-terminal Rb binding domain, also can bind PcG proteins [113,114] and recruit them to E2F target promoters [113]. Together these studies add to the theme that at least some E2F target genes are subject to long-term and possibly heritable repression.

3.4. Chromatin remodeling in Rb-mediated repression

The effects of nucleosomes on gene expression can also be influenced by non-covalent modifications that create localised changes in DNA-histone contacts and/or mobilisation of the nucleosomes on the chromatin fiber to change their relative spacing and positioning on the promoter. These processes, called chromatin remodeling, are carried out by multi-subunit complexes with

an ATP-dependent helicase at the core [115]. There are three families of chromatin remodeling complexes organised based on the similarities of the ATPase subunits. They can regulate gene expression both positively and negatively.

Rb can employ the chromatin remodeling complexes to inhibit proliferation and repress E2F [116,117]. Rb can bind to Brg1 and Brm, two human homologs of the yeast SWI2/SNF2 ATP-dependent helicases, and recruit them to cyclin A (but not cyclin E) promoter [112] to affect gene expression. Brg1 functions are required for Rb to induce cell cycle arrest [118]. Interestingly, the abilities of Rb to interact with Brg1/Brm and HDACs were regulated differently by various cyclin-dependent kinases [119,120]. Phosphorylation of Rb C-terminus by D type cyclin-dependent kinases releases its interaction with HDACs, which relieves the repression of the cyclin E promoter, but not Brg1/Brm, which keeps the cyclin A and Cdc2 promoters repressed. The expression of cyclin E leads to further phosphorylation of Rb and the Brg1 complex [121], which disrupts Rb interaction with Brg1/Brm resulting in expression of cyclin A and Cdc2 genes. These sequential steps, if they can be confirmed with endogenous proteins, may provide a mechanism for the ordered expression of cyclin E and Cyclin A along G1 to S and then to G2/M phases.

Functions of the Brg1/Brm chromatin remodeling complexes are frequently disrupted in cancer cells [122]. The INI1 subunit of the Brg1/Brm complexes is mutated by bi-allelic deletions or truncations in most cases of malignant rhabdoid tumour, a very aggressive pediatric cancer. Brg1 is mutated or absent in a number of human cancer cell lines although the existence of Brg1 mutations in primary tumours has yet to be examined. In the mouse, knockout of either INI1 or Brg1 lead to embryonic lethality. INI1 heterozygous mice develop soft-tissue sarcomas with features of rhabdoid tumours with the loss of the wild type allele [123–125]. Brg1 heterozygous mice are prone to various epithelial tumours [126]. Re-expression of Brg1 proteins in Brg1 negative cancer cells can induce growth arrest, and, importantly, this activity is dependent on Rb [116].

The functional significance of Rb–Brg1 binding however has recently been called into question [127]. Kang and co-workers reported that Brg1 positively regulates p21Cip1 expression to inhibit cell proliferation and these abilities are independent of Brg1–Rb binding. The reasons for the discrepancy between this study and previous reports are not clear.

3.5. DNA methylation and Rb-mediated repression

Methylation of the cytosine base in CpG sequences has long been linked to transcription silencing in development and cancer [128]. Three DNA methyltransferases (DNMTs) have been identified. DNMT3a and DNMT3b are required for *de novo* DNA methylation in early embryogenesis [129] while DNMT1 is responsible for maintenance of DNA methylation [130]. The mechanisms connecting methylated cytosine to gene silencing is provided by a group of methyl-CpG binding domain proteins (MBDs) [131–133]. These proteins can recruit to methylated DNA histone deacetylation and chromatin remodeling enzymes [134–136]. The functional relationship between DNA methylation and histone deacetylation on endogenous heavily methylated promoters was examined with their respective inhibitors [137]. Treatment with HDAC inhibitor TSA alone was not sufficient to reactivate these promoters. In contrast, pretreatment with 5-aza-2′-deoxycytidine, which induces DNA de-methylation, led to robust activation of these promoters by TSA. This finding provides strong evidence that DNA methylation, like histone methylation, can serve to lock-in the repression status affected by histone deacetylation.

In the process of purifying DNMT1, Robertson and co-workers identified Rb, E2F1, and HDAC1 in complex with DNMT1 [138]. When Rb was tethered onto a reporter promoter either through a GAL4 DNA binding domain, or through E2F1, Rb repressive effects can be enhanced by DNMT1. This effect of DNMT1 is partially abolished by TSA. Deletion of the methyltransferase catalytic domain of DNMT1 also partially reduced its ability to enhance repression. These results suggest that DNMT1 can facilitate Rb repression through methyltransferase-dependent as well as HDAC-dependent mechanisms. Interestingly, attempts to detect increased DNA methylation of the reporter promoter did not succeed. In a more recent report, Pradhan and co-workers directly determined the effects of Rb on DNMT1's methyltransferase activity on hemimethylated oligonucleotides [139]. In this assay, Rb actually inhibited DNMT1 through interfering with DNMT1–DNA interaction. The limitations of this assay are that DNMT1 is not tethered onto DNA by a DNA binding protein and naked oligonucleotides may not adequately represent chromatin.

4. Proliferation regulation by Rb through multiple mechanisms

Although E2F repression has been widely accepted as the mechanism by which Rb suppresses cell proliferation at G1–S transition, it is important to point out that many gaps still exist in our understanding of the roles and mechanisms of Rb–E2F repression. At the same time, experimental evidence implicating other mechanisms for Rb role in proliferation regulation has been accumulating.

First, it is now clear from work in multiple laboratories that, in live cells, it is very difficult to detect the

presence of Rb on the promoters of well-established E2F target genes [40,140,141]. The only exception seems to be the cyclin E gene, which is consistent with the fact that cyclin E expression is deregulated in Rb knockout MEFs while many other E2F target genes are not [25,142]. In contrast, p130 was readily detected on these promoters and many cell cycle regulated E2F target genes are deregulated in p107 and p130 double knockout MEFs. Furthermore, knockout of Rb did not affect the recruitment of E2F4 and HDAC onto E2F target promoters, while p107/p130 knockout completely abolished the recruitment of E2F4 and HDAC in the same experiments [143]. These unexpected results could be explained by the possibility that Rb is recruited to other E2F target promoters that were not included in the small sets of testing promoters in these studies. A more recent study however made this explanation unlikely. Screening of DNA arrays consisting of 15000 promoter fragments (selected E2F target genes and cell cycle regulated genes from previous microarray studies) with anti-Rb chromatin immunoprecipitation fragments failed to show presence of Rb on these promoters in three different growth suppression conditions including serum deprivation, contact inhibition, and p16 activation [144]. A possible explanation for this result then is that Rb may be targeted to promoters in other growth suppression conditions, such as cellular senescence as discussed above [96]. These results may point to a new direction in search for the role of Rb–E2F repression in tumour suppression.

The fact that Rb binds more than seventy other transcription factors [145] also suggests that Rb can be tethered onto other promoters *via* their respective transcription factors. Once tethered, Rb, with its associated chromatin modifying enzymes, can affect repression. A recent report indeed demonstrated that Rb can be tethered onto the Cdc25A promoter *via* Stat3 after its activation by hydrogen peroxide [146]. With this rationale, Wells and co-workers conducted Chip-on-CpG array analysis for Rb [48]. This analysis revealed the presence of Rb on promoters of genes involved in embryogenesis and differentiation, metabolism, signalling, as well as oncogenes and tumour suppressor genes. The use of CpG island array still excludes approximately 50% of human genes that are regulated by CpG-poor promoters [147]. Possible direct Rb target genes yet to be identified could include the Pol I promoters for rRNA [148–150] and some Pol III promoters for tRNA and certain small rRNA [151–153].

Second, the final outcome of repression is determined not only by the rate of transcription, but also by a myriad of post-transcriptional mechanisms of regulation, a prominent one being regulation of protein degradation. Ji and co-workers recently showed that although the repression of cellular E2F target genes by timed Rb re-expression followed the same kinetic as the establishment of G1–S arrest, the actual reduction of protein levels of these E2F target genes lagged behind the G1–S arrest by at least six hours [154]. Thus, at least in this experimental system, repression of E2F is inadequate to explain Rb-mediated G1–S arrest. Ji and colleagues went on to show that Rb arrests the cell cycle by inhibiting SCF-Skp2 mediated ubiquitination and degradation of p27Kip1, a cyclin-dependent kinase inhibitor, leading to the inhibition of the kinase activities of cyclin-dependent kinases before their protein levels declined. In fact, p27Kip1 is also required for Rb-induced senescence-like phenotype [20], suggesting that even in the longer term (it takes about two weeks for the senescence-like phenotype to develop after Rb re-expression), when the effects of E2F repression should have taken effect, the role of p27Kip1 is still required.

Third, the involvement of other factors in Rb function has been clearly implicated by genetic evidence. The strongest evidence was provided by the naturally occurring partial-penetrance mutation that changes the arginine at position 661 to a tryptophan, RbR661W mutant [155]. Carriers of this mutation can be disease free, with benign retinomas that spontaneously regress, or with infrequent retinoblastomas that are unilateral (as compared with bilateral retinoblastomas in carriers of null mutations), suggesting that RbR661W still contains significant tumour suppression activity. In various assays, RbR661W was indeed capable of suppressing cell proliferation. Yet, RbR661W was unable to interact with and repress E2F, demonstrating that Rb can suppress cell proliferation through E2F-independent mechanisms [21,154,156]. Indeed, RbR661W retains full ability to interact and inhibit Skp2 to stabilise p27Kip1, which directly leads to G1–S arrest with the same kinetics as wild type Rb [154]. R661W can also function as efficiently as wide type Rb in inducing the formation of PML nuclear bodies [157] and suppressing Ras signalling [158,159]. From another angle, Lomazzi and co-workers showed that the activation of E2F target genes through enforced expression of E2F1 was insufficient to induce S phase entry in serum starved human and mouse primary fibroblasts. Only after Rb was inactivated, did E2F gain the ability to induce S phase entry, suggesting that Rb must be suppressing a molecule(s) other than E2F to inhibit S phase entry [160].

One of the Rb targets may be the DNA replication machinery itself. Rb physically interacts with the DNA replication licensing factor MCM7 [161], replication factor-C (RF-C, [162]), and the single-stranded DNA binding protein Pur α [163]. Through these interactions, Rb may reside on or near DNA replication origins to negatively regulate initiation and/or elongation of DNA replication. Kennedy and co-workers showed that Rb co-localises with sites of DNA synthesis at perinucleolar regions at the start of S phase entry [164]. With the progression of S phase, BrdU positive sites multiplied and

become more broadly distributed but Rb could not be detected at these sites. When cells were cultured in environments that restrict S phase progression, DNA replication sites remained associated with Rb and did not multiply and redistribute [165]. These results, although subject to dispute [166,167], imply that Rb plays a role at the site of DNA replication initiation, which is overridden when DNA replication progresses. Avni and co-workers provided more direct evidence for Rb involvement in DNA replication regulation by showing association between Rb and DNA fragments of four known replication origins through anti-Rb chromatin immunoprecipitation. Importantly, this association was observed after DNA damage and occurred on replication origins in the order they normally fire [168], implicating a role of Rb in restricting DNA replication initiation in response to various signals that activate Rb function through its dephosphorylation. However, *in vitro* DNA replication assays using *Xenopus* egg extracts failed to demonstrate a direct inhibitory activity of Rb [167]. More studies are clearly needed to understand the relationship between Rb and the DNA replication machinery.

5. Rb-mediated stimulation of transcription and cellular differentiation

As a transcription regulator, Rb can also stimulate transcription. Interestingly, almost all the transcription factors that have been shown to be stimulated by Rb are differentiation regulators that specify a particular type of cellular differentiation, such as the myogenic transcription factor MyoD and MEF2 [169,170], adipocyte differentiation transcription factor C/EBPs [171], osteogenic transcription factor Cbfa1/Runx2 [22], melanocyte transcription factor Mitf [172], macrophage differentiation regulator PU.1 [173], and nuclear receptors such as the androgen receptor [174–176]. For these interactions, the recruitments of Rb to the osteocalcin and osteopontin promoter DNA through Cbfa1 [22], and to the tyrosinase and p21Cip1 promoter DNA through Mitf [172] were readily demonstrated (in comparison to the difficulties in detecting Rb association with E2F target promoters). Since more proliferation and less differentiation are both hallmarks of cancer cells, the differentiation-promoting functions of Rb are likely as important as its proliferation suppression functions. This dual-function property (suppressing proliferation and promoting differentiation) of Rb also provides a potential molecular basis for the coupling of proliferation and differentiation [177]. Furthermore, since the differentiation-promoting functions of Rb are cell type specific, they may explain why Rb mutations in cancer have tissue-specific patterns.

Mechanisms for the transcription stimulation functions of Rb are poorly understood. It has been proposed that the differentiation-stimulating functions of Rb could be a secondary effect of proliferation inhibition by E2F repression. In Rb knockout cells, cell cycle exit and early parts of the myoblast and osteoblast differentiation programs can still take place normally, but late differentiation events and permanent cell cycle exit are impaired [22,178–180]. Rb, therefore, may stimulate expression of terminal differentiation genes through, at least in part, establishing a compatible terminal proliferation arrest. A recent study however directly challenges this notion by providing evidence that knockout of Rb after terminal differentiation status was established (formation of myotubes) greatly down-regulated late muscle-specific gene expression but did not allow cells to reinitiate DNA synthesis [181]. Thus, after the establishment of a terminally differentiated state, Rb can be dispensable for maintenance of proliferation inhibition but still indispensable for expression of late differentiation genes. This finding is consistent with earlier mutagenesis studies with Rb, which indicated that E2F repression and stimulation of differentiation are genetically separable functions of Rb [21].

Rapid improvement of our understanding of the mechanisms of Rb-mediated repression has made it more conceptually challenging to understand how Rb can stimulate transcription while interacting with chromatin modifying enzymes that all repress transcription. In this respect, a recent report provided an interesting link between Rb–HDAC interaction and its activation of MyoD [182]. It was previously shown that MyoD is repressed by its association with HDAC1 in undifferentiated myoblasts [183]. Puri and co-workers found that after induction of differentiation, Rb became hypophosphorylated and bound HDAC1 to a degree that about 70% of the cellular HDAC1 could be depleted from cell lysates after immunoprecipitation of Rb. This caused the disassembly of MyoD–HDAC complex and the activation of muscle-specific gene expression by sequestering HDAC1 from MyoD. Indeed, a single mutation in Rb that prevents it from binding HDAC1 abolished its ability to disrupt MyoD–HDAC complex and promote muscle gene expression. In this model however, direct interaction between Rb and MyoD was not required, which raises the question of how Rb can activate differentiation transactivators in a specific manner.

Another mechanistic explanation for Rb ability to activate differentiation transcription factors is provided by physical interaction between Rb and Id2. Id2 heterodimerises with various helix–loop–helix transcription factors and inhibits these transcription factors from binding to their respective promoters. The functional importance of the Rb–Id2 interaction was clearly demonstrated by the rescue of embryonic lethality of Rb knockout embryos with combined knockout of Id2

[184]. Iavarone and coworkers recently demonstrated a molecular mechanism for the rescue of erythropoiesis in Rb deficient embryos by combined Id2 knockout [173]. It was found that Rb-deficient macrophages are defective in supporting the maturation of erythroblasts. The macrophage differentiation master regulator PU.1 activates the expression of CSF1-receptor, which is required for normal development of macrophages. This activity of PU.1 is antagonised by Id2 through heterodimerisation. Rb can activate PU.1 transactivation activity by binding to Id2 and preventing it from binding to PU.1. Rb can also directly bind PU.1 [185] but this binding itself did not seem to be an activating event [173].

Unbiased biochemical purification was recently used to identify Rb interacting proteins in *Drosophila* embryos [186]. The purified complexes did not contain known chromatin modifying enzymes such as HDACs. Rather, of the eight proteins identified in the complexes, seven are related to the *C. elegans* synMuv class B genes. They include the *C. elegans* holomologs of Rb, E2F and DP and other proteins of unknown biochemical functions. In *C. elegans*, major functions of Rb, E2F, and DP are believed to involve regulation of vulva differentiation through gene repression [187–189]. Combinations of mutations in group B genes with mutations in group A or group C synMuv genes induce a multi-vulva phenotype due to inappropriate activation of the vulva differentiation program. In *Drosophila* S2 cells, knockdown of the *Drosophila* synMuv homologs Mip130/TWIT and Mip120 derepressed the expression of genes in sex and differentiation programs. The mammalian homolog of one of the recently identified MuvSyn protein Lin-9 was found to bind Rb and synergise with Rb to stimulate transactivation activity of Cbfa1 [190]. Importantly, lin-9 did not have any effects on Rb ability

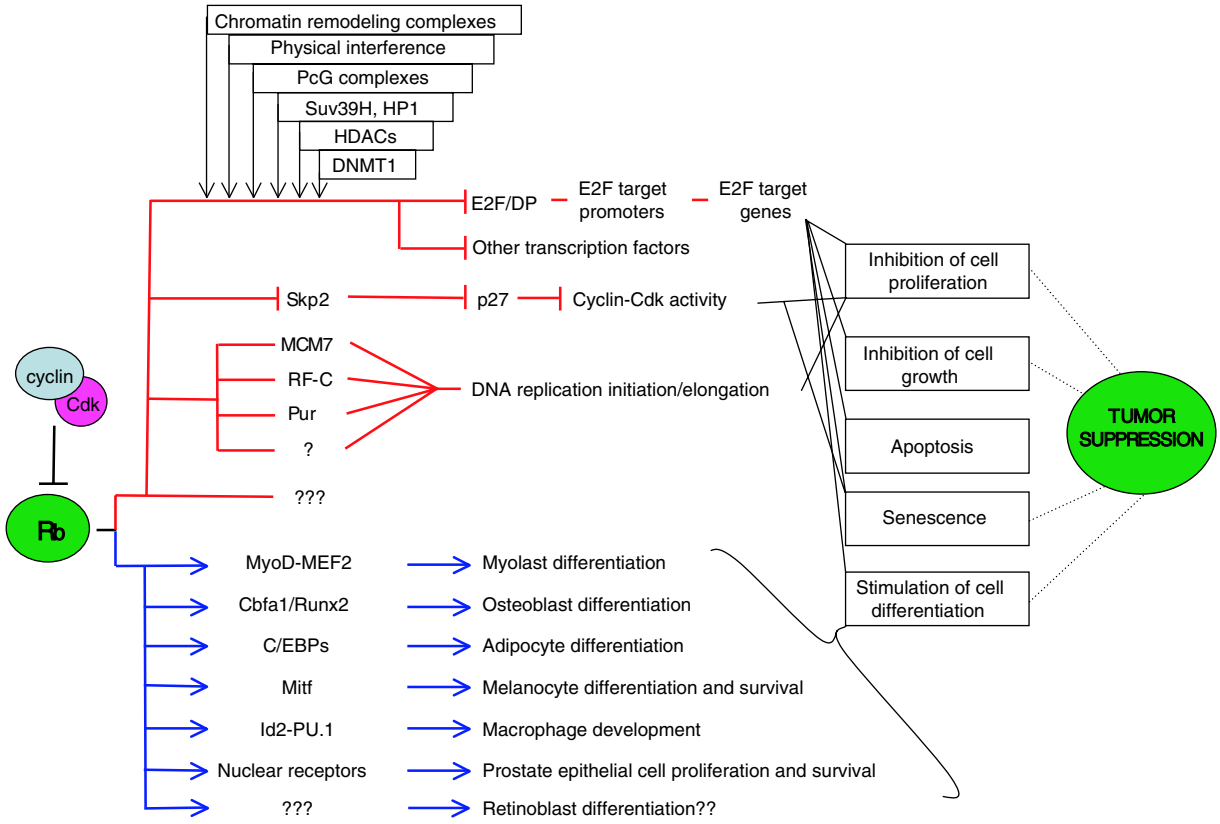

Fig. 1. A network of events regulated by Rb. Rb is believed to function as a transducer between the cell cycle machinery and various cell regulatory mechanisms. Rb function can be generally divided into two categories: inhibition of cell proliferation and stimulation of cell differentiation, represented by red and blue pathways, respectively. The best understood function of Rb is repression of E2F, which involves various chromatin modifying enzymes that Rb recruits to E2F target promoters (top of the Figure). Other mechanisms of Rb-mediated inhibition of proliferation include the Skp2–p27 pathway, the DNA replication machinery, and yet-to-be identified effectors. New findings indicate that Rb–E2F repression affects a broad spectrum of cellular functions in addition to cell proliferation. The ability of Rb to stimulate various differentiation transcription factors may underlie the observed tissue-specific pattern of Rb mutation in cancer. The mechanisms for this Rb function are not known. In this regard, it is still not understood why human retinoblasts are the most susceptible to Rb loss. The dashed lines connecting various Rb-regulated cellular events to tumour suppression connote the incomplete nature of our understanding of their respective roles in Rb-mediated tumour suppression. One of the cellular event, apoptosis in response to Rb loss, is actually counter-intuitive, although it can be considered a cellular safeguard mechanism against tumourigenesis necessitating disruption of the apoptosis response in the multi-step progression towards cancer. It is possible that cellular targets of Rb that remain to be identified may play important roles as well.

to repress E2F or to arrest cell proliferation. Together, these tantalising results suggest the existence of a group of co-factors that function together with Rb to activate differentiation transcription factors.

6. Conclusion

Studies of Rb as a transcription regulator have provided the best understanding of the functional mechanisms of this tumour suppressor. The elucidation of the mechanisms responsible for Rb-mediated repression of E2F has formed a solid framework for the next phase of studies to fill the remaining gaps. Why does Rb seem to govern far less of the established E2F target genes than the other pocket proteins p107 and p130 and yet clearly plays a far more important role in tumour suppression than p107 and p130? How is Rb targeted to E2F promoters in senescence-like states but not in many other forms of proliferation inhibition? Is this distinction relevant to the tumour suppressor function of Rb? What are the roles of the many newly identified E2F and Rb target genes in Rb-mediated tumour suppression? Perhaps the most challenging task is to understand how Rb can function as a transcription repressor on some genes but as an activator on differentiation genes. Since cell type specific differentiation may form the basis for tissue-specific patterns of Rb mutation in cancer, understanding how Rb activates specific differentiation programs may hold the necessary key to tissue-specific therapeutic targets. Finally, it is increasingly clear that Rb uses multiple mechanisms to suppress proliferation; some of them may not immediately impact on transcriptional regulation. A full understanding of Rb must be a multi-faceted one (Fig. 1).

Conflict of interest statement

None declared.

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