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Cellular mechanisms of tumour suppression by the retinoblastoma gene

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Abstract

The retinoblastoma (*RB*) tumour suppressor gene is functionally inactivated in a broad range of paediatric and adult cancers, and a plethora of cellular functions and partners have been identified for the RB protein. Data from human tumours and studies from mouse models indicate that loss of RB function contributes to both cancer initiation and progression. However, we still do not know the identity of the cell types in which RB normally prevents cancer initiation *in vivo*, and the specific functions of RB that suppress distinct aspects of the tumorigenic process are poorly understood.

The initial functional characterization of the retinoblastoma protein (RB) following the seminal discovery of the *RB* gene as the first tumour suppressor focused on its role as a central regulator of cell cycle progression. RB tumour suppressor function was originally thought to be largely due to its capacity to arrest cells in G1 by inhibiting the activity of E2F transcription factors[1,2]. It is now believed that RB has many cellular roles in addition to serving as a G1 checkpoint, including control of cellular differentiation during embryogenesis and in adult tissues, regulation of apoptotic cell death, maintenance of permanent cell cycle arrest and preservation of chromosomal stability[3,4]. Recent studies have also demonstrated that control of the stability of the p27 cell cycle inhibitor (which is encoded by *CDKN1B*) by RB, through the interaction of RB with the anaphase-promoting complex/cyclosome (APC/C), is an important part of the capacity of RB to arrest cells in G1; therefore, E2Fs are not the sole mediators of the capacity of RB to control the G1–S transition[5,6]. In mammalian cells, RB belongs to a family of three proteins that also includes p107 and p130, which are structurally and functionally related to RB and belong to the same cellular pathway, but display distinct functions from RB in specific contexts[4,7–10] (BOX 1). RB is now viewed as a transcriptional co-factor that can bind to and either antagonize or potentiate the function of numerous transcription factors[11,12]. Furthermore, RB is also an adaptor protein that recruits chromatin remodelling enzymes to control the expression of specific target genes and to modify chromatin structure at a chromosome-wide level[13,14] (FIG. 1).

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Overall, although many cellular functions and binding partner proteins have been identified for RB, it remains unclear which of these functions are necessary or crucial for suppressing cancer, and several outstanding gaps remain in our understanding of the mode of action of RB in cells. The goal of this Review is to highlight the potential tumour suppressor role of the known cellular functions of RB, including and beyond its role at the G1–S transition. These functions might each be of particular importance at distinct steps of tumorigenesis — initiation, progression and invasion. Moreover, different functions of RB might be needed to suppress tumour formation in different cell types, such as stem cells and differentiated cells. Understanding unique cell type-specific and tumour stage-specific functions of RB might explain why it is such a potent tumour suppressor in humans, and will potentially provide the knowledge necessary to design novel targeted therapeutics against *RB*-deficient cancers.

Loss of *RB* function and cancer initiation

RB was originally identified through the pedigrees of families whose children developed retinoblastoma. Although tumour penetrance and progression can be under the control of several other factors and genetic events, it is well-accepted that loss of *RB* is the initiating event in these familial retinal tumours, as well as in sporadic cases[15–17]. Loss of *RB* also increases the risk of osteosarcoma development in children and teenagers[18–20]. In adults, human papillomavirus (HPV) is thought to initiate cervical carcinoma and squamous cell carcinoma of the head and neck in part by inactivating RB through expression of the E7 oncoprotein[21,22], and similar mechanisms are possibly involved in virus-induced liver cancers[23]. *RB* is inactivated in more than 90% of human small-cell lung carcinomas (SCLC), and mouse genetic studies have confirmed that RB is crucial in preventing the initiation of this lung cancer sub-type[24] (TABLE 1). Finally, upstream regulators of RB have been involved in cancer initiation in patients, both in familial and sporadic cases, and in several organs and tissues[25–27]. These observations in humans are corroborated by experiments with genetically modified mice: the high penetrance of pituitary and thyroid tumours in $Rb^{+/-}$ mice and the development of other tumours in mice that have been subjected to tissue-specific *Rb* deletion using the Cre–lox system demonstrate that loss of *Rb* is a causal event in many cancer types[7].

Although strong genetic evidence indicates that the *RB* pathway prevents cancer initiation in multiple cell lineages in humans and mice, it is still not clear how and in what cell types cancer initiation occurs on loss of RB function. Generally, adult tissues are composed of stem cells, progenitor cells and differentiated cells (FIG. 2). Stem cells are often quiescent but have a strong regenerative potential and the ability to self-renew. When needed, these stem cells give rise to transit amplifying progenitors, which are actively cycling but lack a significant self-renewing capacity. These progenitors eventually cease cycling and undergo a maturation process that results in differentiated, non-cycling cells.

Loss of RB function and cell cycle re-entry in quiescent stem cells.

Stem cells share many characteristics with tumour cells, including their strong regenerative potential. However, although stem cells have the ability to proliferate and renew, they are more often held in a quiescent state and, unlike cancer cells, only cycle rarely. Maintenance

of quiescence in stem cells is necessary to maintain tissue homeostasis, and the observed role of RB in maintaining quiescence in cell culture models suggests that *in vivo* control of quiescence in stem cells might be a crucial tumour suppressing function of *Rb*.

The role of RB in the maintenance of quiescence in stem cells *in vivo* is largely unknown. Whereas p130, rather than RB, binds to the promoters of genes in G0 cells[28–30], acute loss of *Rb* results in cell cycle re-entry from quiescence in mouse embryonic fibroblasts in culture[31]. *In vivo*, data from several groups indicate that loss of *Rb* in haematopoietic stem cells (HSCs) does not increase their proliferation, although *Rb*-mutant HSCs that are induced to enter the cell cycle under conditions that induce cellular stress might not be able to properly re-enter a quiescent state and can undergo enforced differentiation[32–34]. Deletion of *Rb* in the skin results in a decrease in the number of label-retaining, slowly cycling cells in the stem cell compartment[35]. These data raise two different possibilities: first, RB-mutant skin stem cells might die or differentiate more than controls. Second, these mutant epidermal stem cells might abnormally enter the cell cycle, diluting the BrdU (bromodeoxyuridine) signal; this potential hyperproliferative phenotype might or might not be accompanied by a loss of self-renewal. In addition, the cell autonomy of this phenotype with a decreased number of label-retaining cells remains unknown. Surprisingly, the clearest evidence that RB functions to maintain quiescence in stem cells comes from observations in plants. In *Arabidopsis* roots, loss of the *RB* homologue *RBR* leads to expansion of stem cells in the stem cell pool, seemingly without affecting their self-renewal potential and their proliferative status but by preventing their differentiation[36]. It is interesting to note that if loss of RB function leads to exit from quiescence and an increased number of stem cells without loss of self-renewal capacity, controlled and transient inactivation of RB function in adult stem cells could become a tool to exploit the regenerative potential of these cells in patients.

To date, not enough evidence is available in mammalian cells to conclusively show that loss of RB is sufficient to force stem cells to exit quiescence permanently and whether this cell cycle re-entry phenotype is related to cancer initiation. However, RB is a strong candidate to regulate quiescence in stem cells, and even a slight increase in the proliferation of largely quiescent stem cell pools on loss of *RB* might be sufficient to increase the probability of cancer development as an organism ages — a model that will need to be thoroughly tested.

Cell cycle re-entry in post-mitotic differentiated cells mutant for RB function.

The self-renewal properties and proliferation potential of stem cells and some progenitor cells resemble those of cancer cells. By contrast, fully mature, differentiated cells are thought to have little or no proliferative potential (FIG. 2). Therefore, the probability that a fully differentiated cell re-enters the cell cycle and becomes cancerous might seem low compared with the malignant potential of stem cells. However, post-mitotic differentiated cells largely outnumber stem cells in adult organs and tissues, and evidence indicates that loss of RB might initiate cancer by allowing fully differentiated cells to re-enter the cell cycle. Given the analogy between ‘permanently’ arrested differentiated cells and senescent cells[37,38], the reversal of cell cycle arrest upon acute loss of RB in senescent cells[31,39] offers initial evidence for this model. RB has also been shown to participate in and potentially

modulate the structure and location of heterochromatin formation in senescent cells[40], and loss of RB function might result in chromatin remodelling in differentiated cells, allowing the expression of cell cycle genes and resulting in the de-differentiation of the mutant cells[41,42]. Further support for this model comes from the observation that loss of *Rb* results in cell cycle re-entry in mature hepatocytes[43]. In neurons, however, loss of RB, or even inactivation of the entire *Rb* gene family, does not allow these cells to re-enter the cell cycle[44]. In muscle cells, loss of *Rb* function can result in no cell cycle re-entry[45,46] or in cell cycle re-entry followed by a block in G2 (REF. 47), depending on the culture conditions. In the cochlea, *Rb* deletion allows increased proliferation of precursor cells, and although mature mechanosensory hair cells are capable of re-entering the cell cycle, they fail to complete cellular division[48].

Therefore, cell cycle re-entry in terminally differentiated cells owing to loss of RB function might be context-dependent and most of the time insufficient to initiate cancer. Nevertheless, cells that lose RB function before cell cycle exit and retain the ability to differentiate might also exhibit an increased capacity to re-enter the cell cycle and initiate cancer in the presence of certain stimuli (see below). This idea was suggested by several previous studies[49–51] and was recently illustrated in the retina of mice genetically engineered to develop retinoblastoma[52]. Because it is difficult to imagine how both alleles of the *RB* gene can become mutated in non-cycling cells *in vivo*, this model in which cells lose RB function before they differentiate and are induced to re-enter the cell cycle after they differentiate might be more representative of what is happening during cancer initiation in differentiated cells.

Loss of RB function in cycling progenitor cells and differentiating cells.

Although cancer initiation from quiescent stem cells and post-mitotic differentiated cells upon loss of RB can take place, loss of RB function is more likely to initiate cancer from cycling progenitor cells. In these cells, loss of RB might accelerate proliferation and prevent normal cell cycle exit in G1, which is associated with differentiation (FIG. 2). In support of this model, in HPV-induced cervical cancers, the virus initiates cancer in dividing cells in the basal layer of the epithelium, preventing their normal cell cycle exit and maturation[21]. In the brain, RB levels increase as progenitor cells normally undergo cell cycle exit and loss of *Rb* results in delayed cell cycle exit in these cells[44,49,53,54]. Similarly, *Rb* inactivation in mouse retinal progenitors results in cell-autonomous defects in cell cycle exit, specific differentiation defects, proliferation in differentiating cells and increased cell death. Although loss of *Rb* alone is not sufficient for brain cancer or retinoblastoma development in mice, similar phenotypes are observed in *Rb*;*p107* double-deficient animals that develop retinoblastoma, indicating that failure to exit the cell cycle properly as they differentiate is a mechanism by which mutant *Rb* cells can begin to become tumour cells[50,51,55–57].

In this model, loss of RB might also decrease the ability of cells to differentiate. The role of RB in promoting cellular differentiation during embryonic development and in adult lineages has been well characterized in mutant mice and *in vitro* systems[20,42,58–61]. Mechanistically, RB promotes the differentiation of multiple lineages by binding and regulating tissue-specific transcription factors[11,42] and inhibitors of differentiation such as

ID2 and EID1 (REFS 62–65), as well as general transcription factors and chromatin modifiers[66,67]. Whereas E2F transcription factors were previously thought to mediate only the action of RB on cell cycle progression, recent evidence suggests that the activity of these factors also controls cellular differentiation in *RB*-mutant cells[68,69]. A seminal finding that this function of RB might be crucial in tumour development came from the observation that some mutant forms of RB from families with low penetrance retinoblastoma were deficient in E2F binding but retained the ability to induce the differentiation of tumour cells. This suggests that retention of the ability to induce differentiation might be sufficient to prevent fully penetrant retinoblastoma, and similar observations have been made for osteosarcoma[70–72]. The existence of these mutant forms of RB that lack the cell cycle inhibitory function but retain pro-differentiation activity suggests that different regions or modifications of the RB protein mediate its control over cell cycle progression and differentiation (BOX 2).

RB retention and early cancer progression

Despite the clear evidence that RB normally prevents the initiation of retinoblastoma, osteosarcoma and SCLC, it is striking to note that the vast majority of tumour types only display RB alterations later in tumour progression (TABLE 1) and that patients with familial retinoblastoma are not strongly predisposed to a wide variety of other tumours[73]. These observations suggest that there might be a disadvantage to losing RB too early during tumour development in some contexts. Indeed, RB has pro-survival functions that might seem paradoxical to its role as a tumour suppressor.

Loss of RB function increases cell death and DNA repair.

E2F1 activity is an important mediator of p53-dependent apoptosis in response to loss of RB[74,75], and recent evidence suggests that this cell death might reflect a normal function of E2F1 during the DNA damage response. Double-strand breaks activate the kinases ATM (ataxia telangiectasia mutated) and CHK2 (checkpoint homologue 2), which phosphorylates RB at serine 612, resulting in the formation of a RB–E2F1 complex that can inhibit E2F1-specific target genes that are necessary for apoptosis, such as *Apaf1* and *Trp73* (REF. 76). Similarly, DNA damage increases the acetylation of human RB at lysines 873 and 874, which decreases RB phosphorylation by cyclin–Cdk (cyclin-dependent kinase) complexes and therefore increases its repression over E2F1 (REF. 77). Interestingly, among the E2F family, induction of apoptosis is a function largely mediated by E2F1 and its repression is mediated by specific RB and E2F1 protein domains that are distinct from those through which RB interacts with E2F1, E2F2, E2F3 and E2F4 to control cell cycle progression[78]. Nevertheless, E2F3 can also induce cell death in *Rb*-deficient cells *in vivo*[79,80]. Through these mechanisms, the presence of RB in tumour cells might prevent E2F-mediated apoptosis in response to DNA damage, and might prevent the elimination of tumour cells.

RB seems to have a separate function in the ATR (ATM and RAD3-related) pathway, which responds to single-stranded DNA resulting from stalled replication forks and induces DNA repair. In *Rb*-deficient cells, UV-induced lesions fail to induce cell cycle arrest, unlike in *Rb* wild-type cells; however, DNA repair mechanisms are rapidly engaged[81]. In primary *Rb*-null

mouse hepatocytes, cyclob-utane pyrimidine dimers are removed more quickly than in wild-type cells following UV irradiation[82]. In these hepatocytes, E2F activity has been shown to activate *Ddb2* (damage-specific DNA binding 2), a gene that is crucial for DNA repair, and loss of RB might therefore increase the DNA repair activity of mutant cells[82]. However, UV treatment of *RB*-deficient cancer cells results in apoptosis, similar to that seen with other types of DNA damage[83], and an increase in double-strand breaks is observed in $Rb^{-/-}$ mouse adult fibroblasts treated with DNA-damaging chemotherapeutic drugs[84]. These diverse results suggest that the role of RB in the response to DNA damage might depend significantly on cellular context.

In both of these cases, presence of RB seems to be beneficial to tumour progression, which is counterintuitive given the role of RB as a potent tumour suppressor; these observations might be highly relevant to cancer initiation, following the model that DNA damage signals are induced by oncogenic activation in early human tumours[85]. The idea that RB could promote cancer under certain conditions by increasing survival is supported by experiments in transgenic mice expressing a phosphorylation-resistant, constitutively active allele of *Rb* in the mammary gland epithelium. Strikingly, these transgenic mice develop focal hyperplastic lesions and mammary tumours, probably because the survival of differentiated mammary epithelial cells is extended after pregnancy[86].

A related point is that abnormal proliferation that is induced by loss of RB is often accompanied by increased cell death, through p53-dependent and independent mechanisms[49,57,87–91]. Interestingly, it is possible that functional inactivation of RB by phosphorylation or by viral oncoproteins does not induce the same amount of cell death as deletion of *RB*, potentially explaining why mutations in upstream members of the pathway are selected in some cancers[92,93]. For example, loss of INK4A (which is encoded by *CDKN2A* and is also known as p16) or expression of E1A is insufficient to disrupt RB inhibition of E2F1-dependent apoptosis[92,94]. Both decreased INK4A and presence of E1A result in inhibition of protein interactions that are mediated through the RB pocket region, suggesting the structure and/or specific modifications of RB might distinctly regulate individual functions (BOX 2). However, if cancer cells carry a mutation that protects them from apoptosis, such as inactivation of p53, before losing RB, then the tumour will benefit from the loss of the other tumour suppressive functions of RB (described below) and cancer will progress.

Loss of RB function increases autophagy in response to hypoxia.

During tumour progression, solid tumours eventually achieve a size at which the hypoxic environment limits further growth. Autophagy, which is induced in hypoxic conditions, can initially allow survival of a hypoxic cell by enabling metabolism to continue; however, extended autophagy can eventually result in cell death[95]. The role of autophagy in cancer is currently unclear: one hypothesis is that the absence of both apoptosis and autophagy pathways simultaneously might be beneficial to tumour development by promoting the survival of abnormal cells that accumulate mutations, enabling faster progression to a more malignant phenotype[96]. Recent work has demonstrated that the presence of functional RB might prevent autophagy in response to hypoxia through its inhibition of E2F target

genes[97,98]. So, similar to apoptosis, loss of RB might increase autophagy-mediated cell death, and presence of RB might be beneficial to early tumours under some conditions, although more studies are required to better understand the role of RB in autophagy.

Loss of RB function and cancer progression

Expression and loss of heterozygosity studies indicate that loss of RB function is associated with the progression of human cancers, in addition to its role in cancer initiation (TABLE 1). However, one has to keep in mind that loss of the chromosomal region in which *RB* resides in unstable human tumour cells could be coincidental and not causal. To our knowledge, there is no mouse model yet to specifically test the consequences of loss of *Rb* function during tumour progression, as most current mouse models are based on the simultaneous rather than sequential mutation of cancer genes. Nevertheless, loss of several of the cellular functions attributed to RB might directly participate in tumour progression.

Loss of RB might decrease the differentiation potential of mutant cells.

The early stages of human cancer, such as neoplasia, clearly display proliferation phenotypes while retaining at least some markers of differentiated cells. The degree of differentiation of human cancers is often used to designate the pathological grade of an individual tumour, with the most differentiated being the lowest grade and the least differentiated being the highest grade. Altered RB expression has been observed to be correlated with higher grade, poorly differentiated gastrointestinal cancers[99]. Similar to its role in progenitor cells undergoing differentiation (see above), the presence of RB in tumour cells could in theory promote their differentiation and thereby restrict their proliferative potential, which might explain why RB must be lost during the progression from a well differentiated to a poorly differentiated tumour. Because some anticancer strategies force some tumour cells to undergo differentiation[100], this aspect of RB function might have important therapeutic implications.

Loss of RB results in chromosomal instability.

Human cancers demonstrate a high degree of genomic instability; mutations in *RB* might participate in this phenotype by inducing defects during DNA replication and abnormal chromosomal segregation in mitosis[101–103], which could result in abnormal expression of other cancer genes, enabling tumour progression. Wild-type RB activity might normally slow down the proliferation of tumour cells by acting as a checkpoint not only at the G1–S transition but also during S phase and at the G2–M transition, and loss of this checkpoint could contribute to the ability of a tumour cell to proliferate in the presence of genomic abnormalities. In mouse embryonic stem cells, in which there is no active G1 checkpoint, *Rb* deficiency leads to increased chromosomal alterations[104,105], and in adult mouse fibroblasts loss of *Rb* deregulates S phase, resulting in polyploidy[106]. In normal adult hepatocytes, which are often tetraploid, acute deletion of *Rb* results in cell cycle re-entry and increased aneuploidy[43]. It has been suggested that this type of chromosomal instability is linked to the progression of benign eye lesions to malignant retinoblastoma[17]. Although the mechanisms underlying these aneuploid phenotypes are still only partly understood, RB–E2F complexes are thought normally to restrict the expression of MAD2 (REF. 107), a spindle checkpoint

component. MAD2 levels are increased in *Rb*-deficient cells, correlating with increased levels of activating E2F, and overexpression of MAD2 is sufficient to induce aneuploidy and tumour formation in transgenic mice[108]. In addition, RB might help maintain genomic stability by regulating the expression of, and associating with, chromatin remodelling complexes, thereby regulating chromatin structure, in particular at centromeres and telomeres[109–112]. Many chromatin modifiers bind to RB through the LXCXE binding domain (BOX 2), and recent evidence has demonstrated that an LXCXE mutant that retains E2F-binding capability, and therefore has no effect on MAD2 levels, still contributes to chromosome missegregation and genomic instability through its failure to regulate appropriate pericentric heterochromatin formation[109]. Furthermore, loss of *Rb* can lead to chromosome segregation defects through the misregulation of genes that are important for processes such as centrosome duplication[113,114] and DNA replication[106]. Recent experiments in *Drosophila melanogaster* and human cells also indicate that RB might normally promote chromosomal condensation and preserve chromosomal stability through a direct interaction with a condensin protein[115]. Finally, as mentioned above, *Rb*-deficient cells fail to properly arrest in G1 upon DNA damage and might replicate mutated DNA, leading to the accumulation of mutations[102,105,111,116].

Beyond inducing gross chromosomal changes, loss of RB function might also alter the epigenetic definition of the genome through misregulation of chromatin remodelling enzymes[117] and DNA modifiers. DNA methyltransferase DNMT1, which is both an E2F target[112] and a RB–E2F protein binding partner[118], has been shown to be upregulated in human cancers and in response to loss of RB. Both chromatin remodelling and DNA methylation have been linked to tumorigenesis[119,120]. These epigenetic changes might result in abnormal inactivation of tumour suppressors and activation of oncogenes without the physical mutation of these genes.

Although still poorly understood, the role of RB in maintaining chromosomal stability might be crucial to the prevention of cancer progression.

Loss of RB prevents induction of cellular senescence.

Cellular senescence acts to suppress tumours *in vivo* in response to oncogenic stress, and strong evidence places RB–E2F activity and associated chromatin-regulating complexes, such as SUV39H1, as a key regulators of senescence in cells in culture[121–124]. This is mediated, at least in part, through the formation of senescence-specific heterochromatin at cell cycle gene loci[40]. Furthermore, the RB family controls the length of telomeres and mediates the cellular signals to induce senescence in cells with shortened telomeres[125–127]. Therefore, loss of RB function allows a tumour cell to bypass the cell cycle arrest that is associated with both replication and oncogene-induced senescence during cancer progression. One recent example supports this model: loss of function of the Von Hippel Lindau (VHL) tumour suppressor creates an oncogenic stress that triggers a senescence response both *in vitro* and *in vivo*. This senescent cell cycle arrest depends on the presence of functional RB[128] and is in part mediated by the RB–SKP2 (S-phase kinase-associated protein 2)–p27 pathway. Through this pathway, the presence of RB leads to downregulation

of SKP2 and therefore stabilization of p27, which inhibits cyclin-E-associated kinase activity[129].

Loss of RB promotes angiogenesis.

In order to over-come the growth-limiting effects of hypoxia, tumours select for mutations that increase their ability to recruit endothelial cells to form novel blood vessels in a process termed angiogenesis. Emerging evidence shows that vascular endothelial growth factor (VEGF) and other angiogenic factors that are secreted by tumour cells to recruit endothelial cells are transcriptional targets of the RB–E2F pathway[130]. For example, pituitary tumours arising in *Rb*-mutant mice have high levels of VEGF[131]. Another mechanism by which loss of RB might promote angiogenesis in these pituitary tumours is through the loss of inhibition of ID2 (REF. 132). ID2 is one member of a family of regulators that prevent activity of basic helix-loop-helix (bHLH) transcription factors. Expression of several of the Id proteins has been associated with tumour progression and several genetic studies suggest that the presence of Id factors is necessary for tumour vascularization through regulation of pro-angiogenic factors[133,134]. Additionally, RB might relay receptor-mediated mitogenic signals to develop an angiogenic response[135]. Moreover, deletion of *Rb* in a mouse model of *Trp53*-deficient squamous cell carcinoma demonstrates increased vascularization when compared to similar $Trp53^{-/-}$ tumours[136]. So, although loss of RB might not be sufficient to trigger a full angiogenic response, it might participate in this crucial stage of tumour progression *in vivo*.

Loss of RB function is associated with increased metastatic potential.

Altered RB expression patterns have been observed in poorly differentiated, metastatic hepatocellular carcinomas[137], and correlative analyses have suggested that changes in RB expression are significantly associated with invasion and metastasis of oesophageal cancers[99]. A recent report indicates that loss of RB function is associated with increased levels of cyclooxygenase 2 (COX2) and an increased chance of recurring invasive basal-like breast cancer[138]. COX2 overexpression has been observed in various epithelial cancers and has been demonstrated to increase motility of breast cancer cells in culture[139], as well as invasiveness of colon cancer cells[140]. The correlation between loss of RB and an increase in COX2 expression might reflect a potential mechanism through which RB can prevent metastasis in a tissue-specific manner. Loss of RB has been shown to produce inappropriate migration of neurons in the developing mouse cortex[141], a phenotype that is dependent on E2F3 activity[69]. A similar increase in E2F3 activity in metastatic tumour cells could be indirectly related to the capacity of these cells to be more motile. However, E2F3 inactivation has also been found to enhance the metastatic potential of RB-deficient thyroid carcinomas *in vivo*[142]. More experiments are required to clarify the specific role of E2F3 activity in the metastatic process, but a general role for E2F factors in metastasis is underscored by cell culture experiments, which have identified several E2F target genes with a potential role in invasion and metastasis[143,144]. Additionally, E2F1 over-expression has been shown to increase the invasiveness of human tumour cell lines[145]. Overall, however, our understanding of the molecular mechanisms mediating the potential role of RB in preventing tumour metastasis remains extremely limited.

Conclusions and discussion

In conclusion, a great deal of time and effort has gone into understanding the numerous cellular functions of RB that are mediated by over a hundred known protein binding partners and numerous transcriptional targets (FIG. 3). Some of these functions seem difficult to reconcile with others: RB promotes terminal differentiation and cell cycle arrest in progenitor cells undergoing maturation, but it is also important for maintaining reversible cell cycle arrest in quiescent stem cells that must not permanently withdraw from the cell cycle and differentiate. In addition, many consequences of *RB* inactivation are easily linked to an increase in the tumorigenic potential of the mutant cells, such as loss of a G1 checkpoint or increased genomic instability. But, loss of *RB* might also increase apoptotic and autophagic cell death and the ability to repair DNA lesions[81,84], which might not be beneficial to tumour cells. Although cases have been made for the importance of several of these functions in specific cell types and cancer models, there is still much to be learned about the relative importance of each of these functions in the role of RB as a tumour suppressor *in vivo*. The cellular response to the presence or absence of RB might depend on complex regulatory networks that are poorly understood[146].

It is possible that novel functions for RB remain to be discovered, but it is clear that the crucial importance of the known functions in tumorigenesis need to be further investigated. One method will be to improve mouse models of human cancers that are associated with loss of *RB*[7]. Because loss of *Rb* in mouse models is not sufficient to initiate retinoblastoma, osteosarcoma or SCLC, we do not know if the phenotypes of *Rb*-deficient cells in these organs truly reflect the mechanisms of cancer initiation in the corresponding human cells. Furthermore, it is striking that the mechanisms of cancer initiation in the pituitary, thyroid and adrenal glands, in which loss of *Rb* is sufficient for tumorigenesis, are still largely unknown. When loss of *Trp53* and *Rb* are combined, more tumours develop in mutant mice[24,147–149] but it becomes difficult to dissect the respective roles of these two potent tumour suppressors.

Targeted alterations in upstream components of the RB pathway can result in the inactivation of all of the Rb family genes, but because the RB pathway is not strictly linear, these experiments might not directly inform us about the mechanisms of RB tumour suppressive action[4,7,150–153]. Alternatively, combined deletions in Rb family genes might eliminate the functional overlap within this gene family, but, so far, few models of human cancers have been analysed using this approach. The Van Dyke group has published a series of elegant studies in mice expressing a truncated form of the SV40 large T oncoprotein, T121, which is thought to specifically inactivate the three Rb family members[87,154–160]. These transgenic mice provide powerful models to investigate how loss of Rb family function initiates cancer in multiple cell types, including the brain, the mammary epithelium and the prostate epithelium. These experiments illustrate the point that different cell types respond differently to loss of Rb family members, which could dictate the requirement for specific mutations during tumour progression[161]. In addition, the analysis of mice expressing T121 in the prostate indicates that loss of Rb family function in epithelial cells triggers signals to surrounding stromal cells, underscoring that not all the mechanisms of cancer initiation are cell autonomous[159]. In conclusion, however, although *RB* inactivation is clearly an initiating

event in many cancers, much work remains to be done and new systems need to be developed to better understand how loss of RB function can promote cancer initiation *in vivo*.

The generation of novel conditional alleles of *Rb* and other cancer genes, using a combination of Cre–lox, Flp–frt and tetracycline-inducible systems[162], should enable the production of a new generation of mouse models that can test the consequences of *Rb* loss during tumour progression. These models will also be useful in understanding the continued dependency of a tumour on RB loss, a poorly studied question[163,164]. Similarly, because ectopic expression of RB in cells in culture can produce nonspecific effects, a detailed structure–function analysis of RB *in vivo* will be key to our understanding of RB tumour suppressor functions[78]. In particular, comparing the structure of RB with those of p107 and p130, which are rarely mutated in human cancers and yet are similar to RB in many ways, might help to identify mutant forms of RB that can distinguish specific cellular functions of RB.

Because RB and its upstream regulators belong to a pathway that is often referred to as the linear RB pathway, it is tempting to equate *RB* mutation with *p16* inactivation, activating mutations in *Cdk4* or overexpression of cyclin D1. However, alterations in upstream members of the RB pathway also impinge upon the activities of p107 and p130, as well as other cellular proteins[165,166]. In addition, even high levels of cyclin–Cdk complexes might be insufficient to fully phosphorylate — and functionally inactivate — RB. A clear example that *RB* and *p16* mutations are not equivalent comes from the observations that *RB* is mutated in most, if not all cases of SCLC, whereas *p16* mutations are never found in this lung cancer type but are prevalent in non-SCLC, a different type of lung cancer[167].

A greater understanding of the interplay between Rb family members, regulators of the RB pathway and other binding partners is crucial for the design of therapeutics targeting *RB*-deficient human tumours. Identification of the tumour suppressing functions of RB that are most important within a particular tissue or cell type is necessary to identify compounds that can induce arrest or death of the tumour cells.

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DATABASES

Entrez Gene:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene *Apaf1* | ATM | ATR | *CDKN1B* | *CDKN2A* | CHK2 | COX2 | *Ddb2* | DNMT1 | E2F1 | E2F2 | E2F3 | E2F4 | EID1 | ID2 | MAD2 | p53 | RB | SKP2 | SUV39H1 | *Trp73* | VHL

National Cancer Institute: http://www.cancer.gov brain cancer | cervical cancer | head and neck cancer | liver cancer | oesophageal cancer | osteosarcoma | retinoblastoma | small-cell lung carcinoma

FURTHER INFORMATION

J. Sage's homepage: http://www.stanford.edu/group/sage

Cancer Genetics: http://www.cancerindex.org/geneweb/RB1.htm

Eye Cancer Network: http://www.eyecancer.com

Gene Cards: http://www.genecards.org/cgi-bin/carddisp.pl?gene=Rb1

Gene Clinics: http://www.geneclinics.org/profiles/retinoblastoma/details.html

NCBI Genes and Disease:

http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View.ShowSection&rid=gnd.section.129

OMIM: http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=180200

Retinoblastoma.com:

http://retinoblastoma.com/retinoblastoma

Retinoblastoma genetics: http://www.verandi.de/joomla

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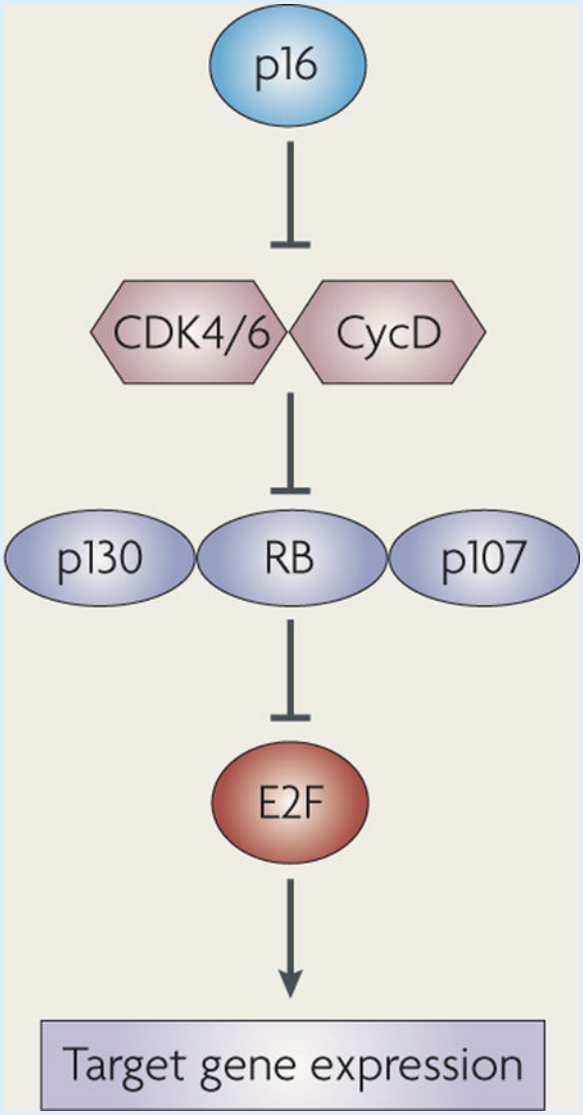
At a glance

- RB, the retinoblastoma protein, has been identified as a crucial tumour suppressor. It is believed to be directly or indirectly inactivated in nearly all human cancers.
- RB has been demonstrated to bind to over one hundred protein partners and has been shown to mediate transcriptional regulation of hundreds of target genes. These protein partners and transcriptional targets are thought to mediate the numerous cellular functions of RB, including temporary and permanent cell cycle arrest, genomic stability, apoptosis and differentiation.
- The cellular functions of RB, as well as a potential role in angiogenesis and metastasis, might contribute to its role as a tumour suppressor, but it is currently unknown which function is most critical. Distinct cellular functions of RB might contribute to its role in preventing tumour initiation versus its role in preventing tumour progression.
- The function of RB that is crucial for tumour suppression might also depend on in which type of cell RB is lost — stem cell, progenitor or differentiated cell — as well as in which tissue.
- In some contexts, presence of RB during earlier stages might be beneficial to tumour progression. Effects of post-translational modifications of RB on individual cellular functions might contribute to preference for a tumour to mutate *RB* or an upstream regulator.

Box 1 |

The Rb gene family and the RB pathway



Three members of the ‘pocket’ protein family exist in mammalian cells — RB (retinoblastoma), p107 and p130. The three family members bind specific subsets of E2F transcription factors[168], and their activity is thought to be largely controlled by phosphorylation. They all contain an LXCXE binding domain to which a number of common cellular partners can bind. In most cell types, overexpressing any Rb family member results in cell cycle arrest in G1.

RB is expressed in both cycling and non-cycling cells, and it seems to be regulated both transcriptionally and post-translationally. Although all three family members bind E2F transcription factors, RB has a unique domain in its C-terminal region that specifically binds E2F1. p107 expression is mostly controlled at the transcriptional level and is expressed in cycling cells. Interestingly, p107 expression often increases after loss of RB.

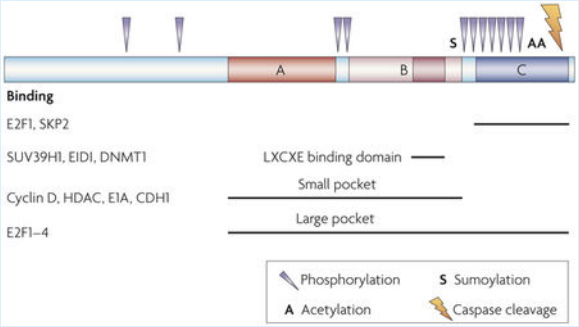
p107 and p130 share a cyclin-binding domain and Cdk (cyclin-dependent kinase)-inhibitor activity. *p130* is thought to be transcribed in all cells but p130 stability is increased in non-cycling cells[169,170].

One major difference between RB, p107 and p130 is that *RB* is commonly mutated in human cancers while *p107* and *p130* are rarely directly inactivated. Instead, when *RB* is not mutated in human cancers, upstream regulators of all three pocket proteins are altered. These upstream regulators, such as p16 and cyclin D, are mutated in a largely mutually exclusive pattern with *RB* mutations, suggesting that these molecules function in a linear pathway to suppress human tumour formation.

Mouse models have demonstrated that E2F is a crucial downstream mediator of the tumour suppressor function of RB; concurrent mutation of E2F1 significantly inhibits the formation of the pituitary tumours and reduces the development of the thyroid tumours that both characterize *Rb*-mutant mice[171]. Surprisingly, activating mutations of the E2F transcription factors themselves are rare in human cancers, suggesting that the tumour suppressor function of RB extends beyond E2F repression[172,173].

Box 2 |

RB structure and post-translational modifications



Human RB (retinoblastoma) consists of 928 amino acids and does not contain any commonly recognized DNA-binding or protein-interacting domains. Deletion and mutagenesis analysis as well as structural studies[174–176] have uncovered several regions that mediate RB binding to individual protein binding partners. Most protein binding partners seem to bind to the pocket region, but some proteins bind unique residues (see figure), making it possible to separate some functions by structure. Furthermore, specific post-translational modifications, which are further described below, might regulate the role of RB in individual cellular functions[78]. The RB C terminus binds specifically to E2F1 and inhibits apoptosis[177]. E1A binding disrupts E2F-dependent proliferation but not apoptosis[92]. Low penetrance retinoblastoma mutations deregulate proliferation but not differentiation[71]. Mutation of the LXCXE binding domain disrupts the ability of RB to mediate H4K20 (histone 4 lysine 20) trimethylation but not proliferation[109].

Phosphorylation

RB can be phosphorylated by several kinases, including cyclin-D–CDK4 (cyclin-dependent kinase 4), cyclin-D–CDK6, cyclin-A–CDK2, cyclin-E–CDK2, CHK2 (checkpoint homologue 2) and RAF1. Phosphorylation of RB enables cell cycle progression, which is thought to occur through hyperphosphorylation-induced release of the E2F transcription factors from the large pocket. Although the binding of many of the partners of RB seems to be disrupted by phosphorylation of RB, it is possible that individual phosphorylation sites might selectively inhibit RB functions; for example, potentially inactivating cell cycle inhibition while allowing apoptosis protection.

Acetylation

Acetylation of RB has been demonstrated to occur at lysines 873 and 874, and this acetylation might prevent RB inactivation by phosphorylation[178]. Acetylated RB might therefore make up a pool of RB that remains active despite the presence of Cdks. Furthermore, double-stranded DNA breaks induce acetylation of RB and acetylation[77] might have a role in differentiation[179].

Sumoylation

RB can be sumoylated at lysine 720, in the small pocket near the LXCXE binding domain, but the function of this sumoylation is unknown[180].

Caspase-cleavage

RB can be cleaved by caspase 8 at the C terminus. Mice lacking the caspase recognition sequence in RB are resistant to apoptosis induced by TNFα (tumour necrosis factor-α) and are more cancer-prone[181].

CDH1, cadherin 1; DNMT1, DNA methylatransferase 1; HDAC, histone deacetylase.

E2F transcription factors

Five members (E2F1–5) of a family of eight mammalian transcription factors that are transcriptional regulators, function as heterodimers with DP1–3 and have been shown to be regulated by direct binding to the pocket proteins.

Transcriptional co-factor

Protein that is recruited to promoters or enhancers of gene expression through binding to other proteins rather than to the DNA itself. Co-factors affect the transcriptional activity of transcription factors.

Cre–lox:

Cre is a recombinase that specifically deletes DNA sequences flanked by *lox* sites.

Autophagy:

A cellular stress response in which cellular proteins and organelles are digested and recycled by lysosomes in order to maintain active metabolism.

Aneuploidy:

The occurrence of extra or missing chromosomes.

Pericentric heterochromatin:

DNA regions around the centromeres of chromosomes that contain hypoacetylated and methylated histones, resulting in transcriptional silencing.

Senescence:

Permanent cell cycle arrest, induced by cellular stresses and telomere shortening. Epigenetic changes prevent a mitogenic growth response, induce a distinct cellular morphology and promote expression of senescence-associated markers. Cells remain metabolically active.

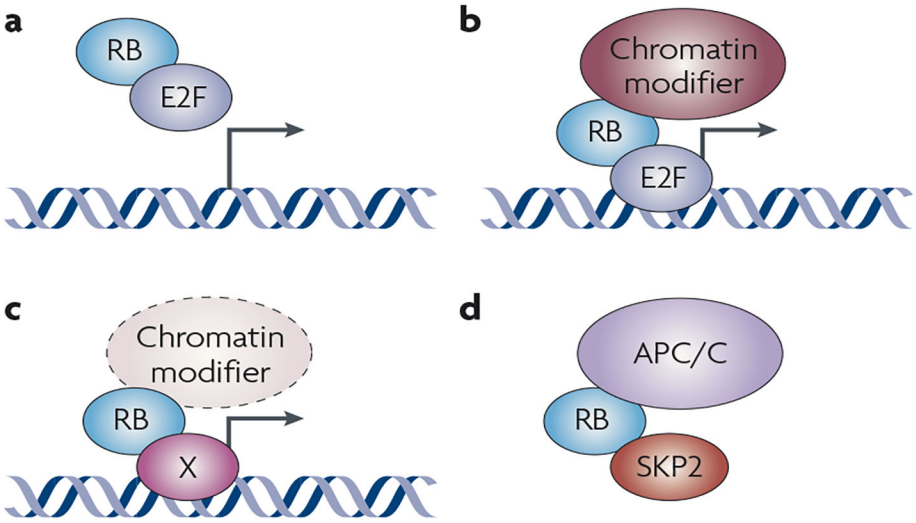


Figure 1 |. RB is a transcriptional co-factor and an adaptor protein that can function through at least four different types of protein interaction.

a | Classically, RB (retinoblastoma) binds to E2F transcription factors and recruits them away from their target genes. **b** | Alternatively, RB is recruited to the promoter of target genes by E2F and inhibits their transactivation activity and further recruits chromatin remodelling complexes (including HDAC (histone deactylase), DNMT1 (DNA methyltransferase 1), HP1A (heterochromatin protein 1A) and SUV39H1) to repress transcription. **c** | RB is a transcriptional co-factor for non-E2F transcription factors or other co-factors, such as the HIF1α (hypoxia-induced factor 1α), MYOD and SP1 transcription factors. **d** | RB serves as a non-chromatin-associated protein adaptor: illustrated is one example of RB acting to recruit APC/C (anaphase promoting complex/cyclosome) and SKP2 (S-phase kinase-associated protein 2) to the same complex, promoting SKP2 degradation.

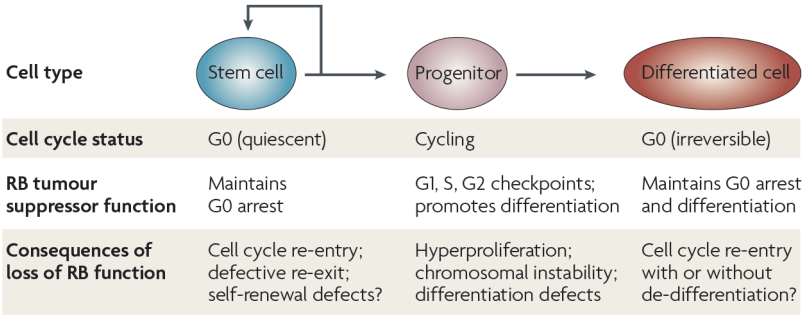


Figure 2 |. Functions of RB that are potentially involved in the prevention of cancer initiation in adult cells.

Adult tissues consist of stem cells that are able to produce cycling progenitor cells; these progenitors produce cells that eventually undergo terminal differentiation. In each of these cell types, RB (retinoblastoma) controls distinct cellular processes, which might be partly E2F-dependent and partly E2F-independent, and might involve extensive chromatin remodelling. Loss of *RB* will have different effects in each cell type. Although the control of the G1–S checkpoint is the most studied function of RB, the abrogation of this function might only be crucial under specific conditions, such as during tumour initiation from cycling progenitor cells.

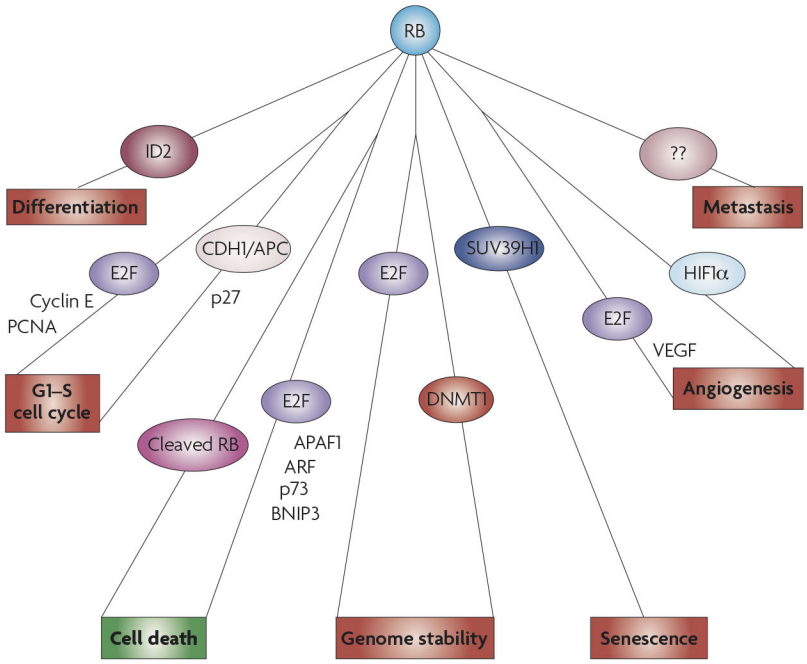


Figure 3 |. Overview of the numerous RB binding partners and transcriptional targets that might mediate its tumour suppressor ability.

Presence of RB (retinoblastoma) might prevent tumour formation by inducing differentiation, controlling cell-cycle arrest, maintaining genomic stability and inducing senescence in response to oncogenic stresses. Furthermore, the absence of RB has been associated with increased angiogenesis and metastasis, although the mediators of these functions are less well understood. Surprisingly, presence of RB has a pro-survival function because of its inhibition of cell death through apoptosis and, potentially, autophagy. This function, shown in green, might be necessary for early tumour cell survival in some contexts. This figure depicts a simplified representation of the potential role of RB in tumour suppression. Each function is illustrated with some of the key protein binding partners and transcriptional targets that might be necessary for the function, but they are not meant to be comprehensive. APAF1, apoptotic peptidase activating factor 1; BNIP3, BCL2-interacting protein 3; CDH1, cadherin 1; DNMT1, DNA methylatransferase 1; HIF1α, hypoxia-induced factor 1α; PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor.

Table 1 |

Alterations of the *RB* gene in common human cancer types

Tumour type	Frequency of *RB* inactivation (genetic or epigenetic)	Presumed consequence of *RB* inactivation	Refs
Lung cancer	Germline *RB* mutations predispose to small cell lung carcinoma (SCLC), and *RB* is inactivated in >90% of sporadic SCLC cases. In contrast, *RB* is mutated in only 15–30% of non-SCLC cases.	SCLC initiation; progression to invasive forms of non-SCLC	182–184
Melanoma	*RB* inactivation is rare in sporadic cases, but inherited mutation predisposes to melanoma	Initiating event in familial cases	185,186
Prostate cancer	~20%	Progression to invasive carcinoma[‡]	187–189
Breast cancer	~20%	Progression	190,191
Bladder cancer	20–50%	Progression to invasive tumours	184,192–194
Leukaemia	Reduced levels of expression are frequent, but mutations in *RB* are rare in leukaemias, except in 20% of chronic myeloid leukaemia (CML) cases	Progression (CML blast crisis)	195–197
Brain cancer	*Rb*-mutant mice develop pituitary tumours, but *RB* mutations are rare in human cases. 15–30% of advanced gliomas have *RB* mutations	Progression	198–201
Oesophageal cancer	*RB* deletion are found in 15–50% of adenocarcinomas or squamous cell carcinomas	Early progression	202,203
Liver cancer	Mutations in *RB* are found in 15–30% of the advanced hepatocellular carcinomas[§]	Progression	137,204–206

[*] Inactivation of *Rb* in the lung epithelial of mice is sufficient to initiate neuroendocrine hyperplasia[216] but the additional loss of *Trp53* is required for SCLC development[24].

[‡] *Rb* deletion in a mouse model is sufficient to initiate prostate cancer[217,218].

[§] Overexpression of Gankyrin in liver tumours might functionally inactivate RB, potentially bypassing the need to mutate *RB* in many cases[219], similar to the expression of E7 by HPV in cervical cancer. Note that cells from common cancers such as colorectal carcinoma[207], pancreatic carcinoma[208], renal cell carcinoma[209], endometrial carcinoma, ovarian carcinoma[210], non-Hodgkin lymphoma[211,212], myeloma[213], thyroid carcinoma[214], HPV-negative head and neck carcinoma[22] and gastric carcinoma[215] only rarely carry mutations in *RB*. However, in many cases, RB is hyperphosphorylated or shows decreased levels in these tumours, and mutations in other members of the RB pathway are usually present.