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Nat Rev Cancer. Author manuscript; available in PMC 2010 August 4.

Published in final edited form as:
Nat Rev Cancer. 2008 September ; 8(9): 714–724. doi:10.1038/nrc2401.

Tailoring to RB: tumour suppressor status and therapeutic response

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Abstract

The retinoblastoma tumour suppressor (RB) is a crucial regulator of cell-cycle progression that is invoked in response to a myriad of anti-mitogenic signals. It has been hypothesized that perturbations of the RB pathway confer a synonymous proliferative advantage to tumour cells; however, recent findings demonstrate context-specific outcomes associated with such lesions. Particularly, loss of RB function is associated with differential response to wide-ranging therapeutic agents. Thus, the status of this tumour suppressor may be particularly informative in directing treatment regimens.

In cancer it is well accepted that tumour cells invoke multiple mechanisms to bypass proliferative control. A crucial junction in the control of cellular proliferation is linked to the retinoblastoma (RB) tumour suppressor protein, whose primary function is to prevent unscheduled entry into the mitotic cell cycle[1–3] (FIG. 1). RB exerts its antiproliferative effects, at least in part, through the ability to mediate the transcriptional repression of genes required for DNA replication and mitosis[4–7]. Through these actions, RB impinges on a sophisticated network of target genes to limit cell-cycle progression[8–11]. Mitogens must counteract this action of RB, and do so through signals that promote activation of cyclin-dependent kinase (CDK)–cyclin complexes, which phosphorylate RB and attenuate its capacity to induce transcriptional repression[12]. Typically, RB remains in this inactive state until passage through mitosis, at which point it is re-engaged through the action of a phosphatase[13,14]. Alternatively, RB action can be invoked during an active cycle in response to specific cellular stresses (for example, genotoxic insult) and induce cell-cycle arrest, thus protecting against continued

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DATABASES
ClinicalTrials.gov: http://clinicaltrials.gov/ NCT00141297
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene API5 | CDK2 | CDK4 | CDK6 | CDKN2A | cyclin D1 | cyclin D2 | cyclin D3 | EGFR | MDM2 | MDM4 | RB1 | p53 | TGFβ1
National Cancer Institute: http://www.cancer.gov/ bladder cancer | breast cancer | cervical cancer | lung cancer | mantle cell lymphoma | oesophageal cancer | parathyroid cancer | prostate cancer | retinoblastoma
National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary/ 5-fluorouracil | Casodex | cisplatin | docetaxel | doxorubicin | etoposide | ICI182780 | paclitaxel | PD 0325901 | PD 0332991 | tamoxifen | UCN01
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inappropriate proliferation1,15,16. Collectively, these functions of RB are thought to be crucial in preventing tumour formation, on the basis of several criteria. First, loss of heterozygosity of *RB1* contributes to tumour formation in the human retina[17,18]. Second, mutations that disrupt RB-mediated transcriptional regulation or genetic events that facilitate RB phosphorylation (for example, amplification of cyclin D1) are frequently observed in tumours[19]. Third, RB inactivation is mediated by and cooperates with oncogenes that contribute to human cancer[20]. These observations support a significant role for RB-mediated cell-cycle control in human tumours and predict that RB deficiency serves to confer a common proliferative advantage. However, recent studies support the hypothesis that the consequence of RB inactivation is quite complex, and can result in disparate outcomes dependent on tumour type. Moreover, it is apparent that different mechanisms used by tumour cells to disrupt the tumour-suppressive function of RB are not synonymous in consequence, suggesting that both tissue type-specific and lesion-specific variances exist with regard to cellular outcome. The findings, reviewed herein, demonstrate that RB inactivation evokes specific responses to cancer therapeutics and suggest that RB status could be developed as a metric to direct therapeutic agents.

RB and cell-cycle control

The contemporary model of RB function in cell-cycle control and tumour suppression is well-founded based on investigation in multiple model systems (FIG. 1). In the absence of mitogenic stimuli, RB activity is engaged to inhibit cell-cycle progression. Although this function of RB can be ascribed to multiple mechanisms, it is clear that RB serves to inhibit the transcription of multiple genes required for S-phase entry[5,7,15,19,21]. The best-studied of these target genes are regulated by the E2F family of transcription factors[1,5–7,22]. In this context, RB mediates transcriptional repression dependent on histone deacetylases, SWI/SNF chromatin-remodelling enzymes and additional chromatin modifiers[1,23–25]. Mitogens reverse transcriptional inhibition of E2F–dependent promoters through sequential activation of CDK–cyclin complexes, which phosphorylate RB and attenuate its transcriptional co-repressor capability[26–28]. The D-type cyclins (cyclins D1, D2 and D3) are considered focal points of this process, as the majority of mitogens signal for D-cyclin accumulation and concomitant formation of complexes between cyclin D and CDK4 or CDK6 (REF 19,REF 29).

Active CDK4 and CDK6 kinases initiate RB phosphorylation[26,30], thus relaxing E2F target gene suppression. Unbiased gene expression analyses revealed that the RB–E2F regulatory targets consist of approximately 150–200 genes[8–11,31–33], many of which are involved in S-phase and mitosis. Within this gene expression programme there is evidence for a feed-forward loop wherein E2F activity stimulates the expression of key factors to activate CDK2 and further promote RB hyperphosphorylation. Thus, mitogenic stimulation initiates an elegant cascade of events to control CDK–cyclin pairing, RB phosphorylation and cell-cycle transition. Once rendered inactive by phosphorylation, RB remains hyperphosphorylated until mitosis, when the regulatory function is restored through RB phosphatase activity[13,14,34]. However, RB action can also be induced during an active cycle in response to anti-mitogenic signals: these return RB to its hypophosphorylated active state through attenuation of cyclin expression, induction of CDK inhibitors or direct modulation of RB by phosphatases[12,15,35–37]. The combined strength and duration of these signals ultimately defines whether RB activity is engaged and whether the overall signalling milieu is permissive for cellular proliferation.

RB pathway and human tumours

From analyses of human tumours, it is evident that RB is disrupted through differential mechanisms to perturb tumour suppressor action. First, amplification and/or overexpression of D-type cyclins is observed in a subset of tumour types[19,38]. Cyclin D1 is a protooncogene

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that is overexpressed as a result of chromosomal rearrangements in parathyroid adenoma and mantle cell lymphoma[19,38]. Additionally, cyclin D1 is overexpressed in a large fraction of breast cancer cases, and mutations of cyclin D1 that enhance its nuclear function are found in oesophageal cancer[39]. Such deregulation of cyclin D1 action is suspected to enhance RB inactivation and thereby promote a proliferative advantage. Second, a similar effect on RB is expected in response to loss of the $p16^{INK4A}$ (*CDKN2A*) tumour suppressor, which is deleted or epigenetically silenced in a large number of human cancers[40,41]. $p16^{INK4A}$ is a true CDK inhibitor: it limits the activity of CDK complexes by binding to CDK4 or CDK6 moieties and disrupting their interaction with D-type cyclins[42–45]. In doing so, $p16^{INK4A}$ also triggers the release of $p21^{CIP1}$ or $p27^{KIP1}$ (which serve to support CDK4 activity but suppress CDK2 activity44,46) from cyclin D–CDK4 and cyclin D–CDK6 complexes. Thus, $p16^{INK4A}$ potently regulates RB phosphorylation both directly (through inhibition of CDK4) and indirectly (through release of CDK2-specific inhibitors). The signals that induce $p16^{INK4A}$ are diverse, including those that elicit cell-cycle exit (for example, catastrophic DNA damage) or paradoxically those that are associated with tumorigenesis (for example, activated oncogenic Ras). In both contexts, upregulation of $p16^{INK4A}$ is considered to be part of a senescence programme that limits inappropriate proliferation and tumorigenesis[47–49]. Consistent with a role for limiting CDK4 activity in the suppression of tumorigenesis, mutations of CDK4 that bypass the action of $p16^{INK4A}$ are observed in specific cancers, as is the amplification of CDK4 (REF 50–REF 52). Lastly, direct perturbations of RB action occur through deletion or mutation of *RB1* itself, as is observed at high frequency in retinoblastoma or small-cell lung cancer[40,53,54]. Additionally, RB is the target of the HPV-E7 oncoproteins involved in the aetiology of cervical cancer[55,56]. Moderate frequency of RB loss has been observed in other tumour types (for example, breast cancer, bladder cancer and prostate cancer), as determined by analyses of loss of heterozygosity or immunohistochemical staining. Thus, disruption of at least one arm of the RB–$p16^{INK4A}$–cyclin D1 triumvirate appears crucial to tumour formation and/or progression[21,40,41,45].

Are all RB pathway lesions equal?

Based on mechanisms of action it was suspected that the differential mechanisms used by tumours to evade RB function resulted in overlapping biochemical and cellular consequence, and would therefore occur as mutually exclusive events in human disease. Evidence for the latter supposition has been documented in human tumour samples. For example, tumour cell lines or primary tumours lacking RB remain proficient for $p16^{INK4A}$, and express low levels of cyclin D1 (REF 21,REF 40,REF 41,REF 45,REF 54,REF 57,REF 58). Not only are these events mutually exclusive, but there is a tumour-selective preference for ablation of any one pathway participant (TABLE 1). For example, within lung cancer, it is apparent that small-cell lung cancer is characterized by RB loss, whereas non-small-cell lung cancer is associated with a higher frequency of $p16^{INK4A}$ loss[54,59,60]. In other tumour types, differing frequencies of $p16^{INK4A}$ loss, RB loss and cyclin D1 amplification or overexpression are observed (TABLE 1). The impetus behind the observed selection of differential genetic or epigenetic alterations between tumour types and the influence on disease outcome is only now beginning to emerge, and is probably attributed to non-synonymous consequences of each genetic lesion. However, homogeneity in the RB pathway is the exception rather than the rule.

Diverse impact of RB dysfunction

It is vital to appreciate that the effect of disrupting the RB pathway is contextdependent and can lead to diverse outcomes (FIG. 2a). For example, deletion of *Rb1* in murine fibroblasts has only a minor effect on cell-cycle kinetics, but can compromise differentiation programmes or lead to genome instability[9,61–65]. Similar observations have been made in other cell culture systems and specific mouse tissues, such as liver, melanocytes and ovary[66–68]. These findings

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contrast with the crucial role for RB in controlling proliferation in the pituitary and several other murine organs (for example, epidermis), with similar results also shown in specific cell culture models[69–71]. Importantly, there are also significant differences in the consequence of RB loss between murine and human models. For example, *Rb1* heterozygosity is associated with pituitary and thyroid cancer in mice, whereas humans with a defective *RB1* gene develop paediatric retinoblastoma followed by increased risk for multiple secondary tumours in adulthood[72–74]. Such heterogeneous results become more complex if one takes into account the disparate effects of $p16^{INK4A}$ loss or cyclin D1 overexpression in culture models and mouse tissues. Thus, it is crucial to consider the overall significance of RB disruption in a manner that is highlight modified by genetic, tissue and organismal context. Crucially, this specificity significantly changes when conditions that inhibit proliferation and mediate the dephosphorylation of RB are considered.

Pathway specificity

Many signals and pathways lead to G1 arrest and the dephosphorylation and activation of RB[1,15]; of these, only a minor subset can be bypassed by cyclin D1 overexpression or $p16^{INK4A}$ loss. There are several proposed mechanisms that underlie these observations. First, relatively few anti-proliferative pathways function through $p16^{INK4A}$ (REF. 75). Second, cyclin D1 levels are often reduced by protein degradation or other mechanisms that are dominant to overexpression and/or amplification of *CCND1* (REF 19,REF 76). In addition, cyclin D1 has been reported to be mislocalized to the cytoplasm in a subset of tumours, thus potentially limiting its effect on the nuclear RB protein77,78. Third, there is evidence that RB dephosphorylation induced by cell stress can emerge through mechanisms other than attenuation of CDK4 and CDK6 activity[12,36,37]. Thus, for many stress responses RB loss is distinct from upstream alterations in the pathway.

From studies using murine fibroblasts it has been shown that specific deletion of *Rb1* alters the cell cycle-inhibitory effect of DNA-damaging agents, transforming growth factor β (TGFβ1) signalling, oncogene signalling and inhibition of the Ras pathway[35,79–81] (FIG. 2b). This effect of *Rb1* deficiency can lead to disparate outcomes that are highly relevant to cancer treatment. For example, *Rb1* deficiency leads to enhanced cell death following genotoxic DNA damage versus ongoing proliferation in the case of Ras inhibition or TGFβ1 exposure[35,82,83]. Thus, RB status can serve as a crucial determinant in bypassing cell cycle-inhibitory pathways and/or promoting cell death.

Cell death

A multitude of studies in cell culture and mouse models support the concept that RB loss is associated with enhanced susceptibility to cell death. Although numerous cell types and mouse tissues can tolerate RB deficiency[84,85], there is also a general predilection toward cell death that is apparent in specific contexts[22,71,86,87]. Two complementary mechanisms are invoked to explain this facet of RB function. First, inappropriate cell cycle progression can sensitize to cell death. A key example of this involves DNA-damaging agents or microtubule poisons wherein deregulated cell-cycle progression can lead to enhanced damage burden by inducing secondary replication-associated lesions or mitotic catastrophe[88]. Second, unrestrained E2F activity can lead to the aberrant expression of pro-apoptotic genes that will predispose RB-deficient cells to death[22,82,89,90]. Consistent with the latter concept, RB loss sensitizes cells to a variety of pro-apoptotic stimuli that do not intrinsically act on the cell cycle[86,87,91,92], and the effects of RB on cell cycle and apoptosis can be dissociated[91]. Additionally, loss of RB can contribute to p53-mediated cell death through a mechanism that is at least partially dependent on the ARF tumour suppressor[93]. ARF expression can be induced by inappropriate E2F activity, as occurs with the disruption of RB, and this triggers activation of p53 and apoptosis[94,95]. As such there can be clear cross-talk between the RB and p53 pathways.

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However, loss of RB also contributes to both ARF- and p53-independent cell death[96–98]. Therefore, an appealing goal is to target signals and pathways that will specifically induce death in ectopically dividing RB-deficient cells.

Senescence

Akin to apoptosis, senescence is viewed as a terminal fate for cells and involves 'irreversible' cell-cycle arrest. Senescence can be induced by a panoply of signals, including activation of oncogenic signals, withdrawal of oncogenic signals, restoration of p53 activity, DNA damage and specific therapeutic regimens, thus serving as an intrinsic barrier to tumorigenesis[47,48,99–101]. $p16^{INK4A}$ is a marker for senescence and is required for the establishment of cell-cycle arrest, whereas RB loss is sufficient to mediate escape from senescence[102,103]. Thus, aberrations in the RB pathway may be particularly relevant to escape the engagement of this pathway.

Although many of these functional findings have been generated using murine fibroblasts *in vitro* or analyses of mouse development, it seems logical to consider whether the status of the RB pathway could influence the response of a tumour to specific therapeutic modalities.

A case for hormone independence

RB pathway aberrations are found with significant frequency in hormone-dependent cancers, including those of the breast[104–108] and prostate[109–111]. As such, several studies assessed the impact of RB status on endocrine-based therapeutic regimens (TABLE 2).

Breast cancer

Breast cancers that are oestrogen receptor (ER)-positive are frequently treated using hormone therapies that deplete oestrogen (such as GnRH agonists) or directly antagonize the transactivation function of ER (such as tamoxifen or ICI182780). The effects of oestrogen antagonists on the cell-cycle response in ER-positive models of breast cancer have been analysed extensively[112–114]. These cell culture studies demonstrated that ER antagonism leads to cell-cycle cessation in G1 and the dephosphorylation and activation of RB. $p16^{INK4A}$ activity seems not to be required, as commonly used ER-positive tamoxifen-sensitive cell lines lack $p16^{INK4A}$ expression[115]. Furthermore, cyclin D1 levels are effectively attenuated in ER-positive cell lines, including those that express high levels of endogenous cyclin D1 (for example, MCF7 cells)[116]. Indeed, overexpression of cyclin D1 is not sufficient to maintain RB phosphorylation or promote proliferation in the face of prolonged exposure to oestrogen antagonists117. Thus, irrespective of upstream lesions in the RB pathway, the RB protein can be activated and cell-cycle progression impeded. Presumably, this is because ER antagonists affect multiple facets of cell-cycle machinery that coalesce in the regulation of RB114,117. In such cell culture models, knockdown of *RB1* by RNA-interference approaches or functional inactivation through the use of viral oncoproteins leads to an effective bypass of the effects of tamoxifen and related therapeutics[118,119]. These studies were extended to xenograft models, where RB deficiency leads to therapeutic failure with tamoxifen[119]. These findings suggest that disruption of RB function, but not loss of $p16^{INK4A}$ or cyclin D1 overexpression, has a deleterious effect on the treatment of ER-positive breast cancer.

The relevance of the RB pathway to tamoxifen response in cancer biopsies has been widely analysed. Although cyclin D1 is overexpressed in a large fraction of ER-positive breast cancers, the relevance of this event to the response to tamoxifen remains uncertain. In several large studies, high-level expression of cyclin D1 was not observed to influence the response to tamoxifen therapy[120–122]; however, other similarly devised studies suggest that overexpression of cyclin D1 is associated with tumour recurrence for tamoxifen therapy[123,

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[124]. This controversy remains unresolved, but could be related to the observation that overexpression of cyclin D1 is associated with a form of breast cancer that has intrinsically improved prognosis[123,125–127]. Surprisingly, overexpression of *p16INK4A* is generally associated with a poor prognosis in breast cancer[128,129]; moreover, loss of $p16^{INK4A}$ has been only been marginally studied in the context of tamoxifen therapy, and appears to hold little prognostic significance[130,131].

Consistent with the lack of clear relationship with upstream aberrations in the RB pathway, hyperphosphorylation of RB, as detected using phospho-specific antibodies, is not associated with response to endocrine therapy[132]. As discussed in more detail below, loss of RB is difficult to evaluate histologically. Furthermore, the percentage of ER-positive breast cancers that lack RB expression is rather limited (10–20%). However, histological loss of RB has been associated with a poor response to endocrine therapy[132,133]. Furthermore, indirect analyses of RB loss, using gene-profiling approaches, further support this concept[31,118]. Thus, combined with the data from preclinical models, there is evidence to suggest that RB loss is associated with therapeutic failure and that heterogeneity within the RB pathway could be of particular significance in specifying response to oestrogen antagonists.

Prostate cancer

Prostate cancers are exquisitely dependent on androgen receptor (AR) activity for growth and progression[134]. Given the poor response of this tumour type to cytotoxic therapeutic agents, strategies to ablate AR function (achieved through the use of androgen depletion strategies or direct AR antagonists) are the first line of treatment for disseminated prostate tumours[135].

In cell culture models of androgen-dependent prostate cancer, such therapeutics lead to cell-cycle arrest in G1 that is accompanied by reduced expression of D-cyclins and efficient RB dephosphorylation[136]. Given these observations, there has been a concerted interest in delineating the mechanisms by which the cyclin D1–$p16^{INK4A}$–RB axis may be perturbed in the transition to androgen independence and thus promote therapeutic resistance. With regard to $p16^{INK4A}$, although overexpression can potently arrest prostate cancer cell lines[137], there is little evidence that $p16^{INK4A}$ induction participates in cell-cycle exit following AR antagonism. Similarly, although D-cyclins are induced by androgen through post-translational mechanisms[138], cyclin D1 expression is not sufficient to restore cellular proliferation in cultured prostate cancer cells challenged with androgen ablation or androgen antagonists[137].In this cell type, accumulated cyclin D1 markedly antagonizes AR function and can impede subsequent rounds of cellular proliferation through kinase-independent mechanisms that have been well defined[139–142]. Thus, cyclin D1 has a general anti-proliferative role in such models. By contrast, emerging evidence suggests that disruption of RB itself may have a significant consequence on prostate cancer therapies. Initially, it was observed that viral oncoproteins with the capacity to inhibit RB function were sufficient to promote cell-cycle progression in the absence of androgen or presence of androgen antagonists[136]. Subsequent analyses of isogenic, AR-positive cancer cells revealed that RB depletion alone rendered no discernable proliferative advantage to prostate cancer cells in the presence of androgen[143]. However, RB depletion in prostate cancer cells was sufficient to sustain cell-cycle progression after challenge with androgen ablation and/or AR antagonist strategies that mimic therapeutic intervention[136,143]. Based on these observations, it was suspected that aberrations in RB itself (rather than loss of $p16^{INK4A}$ or overexpression of cyclin D1) may contribute to hormone resistance.

Despite these findings, clinical studies examining the impact of cyclin D1, $p16^{INK4A}$ or RB as determinants of therapeutic outcome have been only preliminarily considered. $p16^{INK4A}$ loss is infrequently observed; conversely, increased $p16^{INK4A}$ levels are associated with a poor prognosis, similar to that observed in breast cancer[144–146]. Thus, $p16^{INK4A}$ function appears

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to be maintained in the majority of prostate cancers, and $p16^{INK4A}$ loss does not appear to have a role in the transition to androgen independence. A role for cyclin D1 in this process is similarly obscure, and the function(s) of cyclin D1 in this tissue type incompletely defined. Several reports have demonstrated that cyclin D1 expression is rare or infrequent in primary disease, supporting the idea that excessive cyclin D1 expression is probably not a major factor in disease development or progression[147–150]. Furthermore, recent analyses showed that cyclin D1 is low or mislocalized to the cytoplasm in a significant fraction of prostate cancers obtained from radical prostatectomy78. However, a subset of tumours, either associated with high $p21^{CIP1}$ levels or associated with bone metastases, do show enhanced nuclear cyclin D1, suggesting that cyclin D1 function is complex and dependent on molecular milieu[78,149]. Surprisingly, there have been few studies of RB loss in prostate cancer specimens; however, RB loss is overrepresented in recurrent prostate cancers, which are resistant to hormone therapy[109]. Furthermore, the gene expression profile generated by viral oncoproteins that disrupt RB function is associated with poor disease outcome in prostate cancer[31]. These findings are consistent with functional analyses of RB loss in cultured prostate cancer cells, and indicate that RB loss may have a specific role in the acquisition of androgen independence.

Based on these collective findings, it is predicted that the efficacy of endocrine-based therapies for breast and prostate cancers is particularly reliant on RB activation, and that loss of RB serves as a mechanism to bypass therapeutic intervention.

Priming to kill: cytotoxics

Contrary to results observed with hormone-based regimens, preclinical models in a multitude of systems suggest that RB loss can actually sensitize cells to specific cytotoxic or genotoxic agents, but this response is dependent on tissue type (TABLE 2). DNA damage will effectively and rapidly lead to the degradation of cyclin D1 (REF. 76), and RB-dependent DNA damage checkpoints are operable in the absence of $p16^{INK4A}$ (REF 118,REF 151). Thus, upstream lesions in the RB pathway are not *a priori* crucial determinants of the response to chemotherapeutic agents. Whereas breast cancer cells or xenografts depleted of RB can successfully evade ER antagonists, these cells are more susceptible to elimination induced by cisplatin or ionizing radiation than isogenic RB-proficient counterparts[118]. Similar effects were observed in lung cancer xenografts, where RB depletion sensitized cells to the cytotoxic effects of cisplatin, doxorubicin and 5-fluorouracil[151]. In both cases, failure to arrest after chemotherapeutic intervention was noted[118,151]. Interestingly, these effects were observed both in the presence and in the absence of p53, suggesting that RB can function as a determinant of cytotoxic therapies independently of p53. However, these outcomes were not uniformly observed with all tumour types or cytotoxic agents. For example, in the context of prostate cancer, RB-depleted cells were sensitized to cell death induced by microtubule poisons (docetaxel and paclitaxel) and etoposide but, in contrast to breast and lung cancer cells, were notably more resistant to cisplatin[143]. The basis of this divergent sensitivity is a focus of current investigation, and will provide a platform through which to delineate the underpinning mechanisms of divergent chemotherapeutic responses.

The effect of RB deficiency on the response to cytotoxic therapies has also been observed in tumour specimens. Specific loss of RB is associated with improved response to cytotoxic therapeutics in bladder cancer[152,153], and recently published studies have demonstrated that although hyperphosphorylation of RB does not affect the response to cytotoxic agents in breast cancer, RB loss is directly associated with improved outcome with chemotherapy[132]. In this study the effect of RB status was independent of p53 and other pathophysiological markers in multivariate analyses. Thus, loss of RB in tumours could be a specific determinant of sensitivity to cytotoxic therapies.

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Although these findings are promising, it has yet to be conclusively determined whether RB deficiency and resultant sensitivity to cytotoxic agents broadly translates into improved long-term survival. Analyses in RB-deficient lung cancer xenograft models showed that the initial dramatic response to cisplatin and doxorubicin did not lead to a durable effect on tumour burden. Rather, RB-deficient tumours recurred readily with the completion of the treatment cycle[151]. Such a troubling trend is observed in specific tumour types, such as small-cell lung cancer (which often loses RB), where the initial response to therapy can be quite effective but recurrence of resistant disease occurs at a high frequency[154]. Therefore, additional studies will be required to define how RB loss translates into overall survival in additional tumour types following specific treatment regimens.

RB and kinase inhibitors

In addition to hormonal and cytotoxic agents, a number of kinase inhibitors have been shown to depend on RB protein function for activity (TABLE 2). Although not yet in routine clinical use, these agents are viewed as foundations for ongoing therapeutic development or have been used in the context of clinical trials. The kinase inhibitors that have been interrogated fall into three principle categories as discussed below.

Erk/MEK inhibitors

Based on the finding that RB-negative murine fibroblasts can proliferate in the presence of Ras antagonists[79], it is perhaps not surprising that they are less sensitive to the effects of MEK inhibition by U0126. This has been demonstrated in both primary and transformed rodent lines and has been associated with RB status in human tumour lines[155]. Although U0126 is not used clinically, the compounds PD 0325901 and AZD6244, which are in clinical trials, would be expected to function in a similar fashion. Thus, loss of RB may have a significant impact on the response to such agents.

UCNO1 and staurosporine

These agents are broad-functioning kinase inhibitors with targets that include protein kinase C (PKC) enzymes and checkpoint kinases. Irrespective of function, both agents lead to RB-dependent suppression of cell-cycle progression [156–158]. As such, RB loss enables tumour cells to proliferate in the presence of these agents.

CDK inhibitors

Given the central role of RB in cell cycle control, there is an obvious need to determine the impact of RB status on the cellular response to CDK inhibitors. Agents such as roscovitine that target CDK2 activity also target CDK1 and can inhibit cell cycle progression independent of RB. However, consistent with RB being a downstream target of CDK4, RB is required for the effect of the compound PD 0332991, as determined in cell culture and xenograft experiments[159,160]. Thus, different classes of CDK inhibitor elicit distinct dependence on the RB pathway for efficacy.

Combined, the analyses of these agents suggest that RB deficiency could represent a bypass mechanism for a relatively large range of ‘molecularly-targeted therapies’ that function through a cytostatic mechanism.

RB status in clinical application

Given the preclinical indications that RB status might be considered for development of tailored therapy, it is imperative to identify hurdles associated with the development of RB as a prognostic marker. Although genetic consideration of RB status in the context of

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retinoblastoma diagnoses is routine and deletions of the locus at 13q14 are used to define severity of multiple myeloma[161,162], few treatment regimens interrogate RB status when considering efficacy. At present there has been only one National Cancer Institute-sponsored trial that considered the presence of RB for inclusion, which used the CDK4-specific inhibitor PD 332991 (NCT00141297). This agent is critically dependent on RB for tumour-static activity in xenograft models159,160; thus the eligibility criteria are important for the logical direction of therapy. The importance of targeting RB has also been appreciated retrospectively in several studies, where reduced levels of RB phosphorylation (suggesting effective engagement of RB) have been used as a means to assess drug efficacy[163,]164.

To most effectively challenge the contribution of RB for therapeutic response in prospective studies, it must first be determined how RB status might be effectively and economically determined (FIG. 3). While genetic loss of *RB1* would be considered the gold standard, this approach fails to detect the myriad of additional mechanisms through which RB is functionally inactivated in cancer. Traditional immunohistochemical analyses also have limitations, in that RB levels fluctuate as a function of cellular proliferation[165], and the protein can be expressed yet be inactive in tumour cells. These facets of RB protein expression may in part explain the somewhat confused picture of RB status that emerges from histological studies. Although phospho-specific antibodies have been used to assess RB phosphorylation in biopsy specimens[163,164,166,167], as discussed above, the basal state of RB phosphorylation is not necessarily indicative of altered response to therapeutic agents.

Owing to such limitations, it is appealing to use indirect methods for monitoring ‘RB activity’ in tumours. An unexpected marker for loss of RB activity may be high-level expression of $p16^{INK4A}$ (REF. 168) (FIG. 3). The $p16^{INK4A}$ protein is induced by oncogenic stresses to suppress tumorigenesis; however, tumours lacking RB are able to proliferate despite high levels of $p16^{INK4A}$ expression[169,170]. Thus, scoring for $p16^{INK4A}$ and proliferative markers (for example, Ki67) would be expected to define RB-deficient tumours. This combination proved effective in defining ductal carcinomas *in situ* that are likely to progress[168], and could be useful in deciphering the ability of established tumours to respond to therapy. Consistent with this supposition, high levels of $p16^{INK4A}$ in breast and prostate tumours are an indicator of poor prognosis[128,129,171]. Additionally, high levels of $p16^{INK4A}$ are used to define cancerous cervical lesions that arise as a consequence of HPV-mediated RB inactivation[172,173].

An alternative strategy makes use of the known programme of genes that are controlled by RB–E2F[8–11,]31. Although there is some heterogeneity between tissues, the RB gene expression signature has been readily observed in numerous tumour types, including breast, lung and hepatocellular carcinoma31,118,[174]. The RB gene expression signature shares significant features with other gene expression signatures that have been associated with disease outcomes including, the ‘proliferation signature’, the ‘chromosome instability signature’, ‘genomic grade index’ (GGI), and ‘recurrence score’ (Oncotype Dx)[175–]179 (FIG. 4). Of these signatures, the GGI and recurrence score have been shown to have significant utility in differentiating tumours that will respond to tamoxifen therapy or will require more aggressive therapy[176,177,180–186]. Such prognostics are currently being analysed in the context of prospective breast cancer clinical treatment, and the signature of RB–E2F–regulated proliferative genes contributes to the predictive nature of virtually all such gene expression prognostics[176,185]. Therefore, it could be argued that RB activity is already being considered in the context of breast cancer prognosis, and that the recent preclinical investigations may be useful in refining and directing the use of such predictive signatures.

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Exploiting RB loss therapeutically

Directly exploiting the intrinsic properties of RB deficiency to therapeutic end could have utility in a large range of human cancers. Whereas other targets have been the subject of intense screening efforts, only a single unbiased chemical screen that was directed at specific elimination of RB-defective tumour cells has been reported. In this system, RB activity was compromised by viral oncoproteins in an isogenic human transformed fibroblast system[187]. Although viral oncoproteins do efficiently target RB, a caveat of these studies is the impact of viral oncoproteins on additional factors of importance in cancer. These potentially confounding factors aside, in a total of >25,000 compounds screened, the only compounds that selected for RB-deficient cell death were established topoisomerase II poisons (for example, doxorubicin) [187]. Strikingly, doxorubicin has significantly enhanced effect in treating RB-deficient lung tumour xenografts151. Thus, relatively simple chemical screens could be effective in defining compounds that exploit the impact of RB loss, and should be considered for tissue-specific analyses of cytotoxicity as a function of RB status.

An alternative therapeutic approach to target RB-deficient cells may be to harness the latent pro-apoptotic activity of E2F (FIG. 4). One such approach takes advantage of the fact that E2F1 activity, which is particularly pro-apoptotic, is restricted by CDK2-mediated phosphorylation in latter phases of the cell cycle[188,189]. For example, in the absence of functional RB, CDK2 inhibition leads to increased E2F1 function and resultant cell death[189]. Conversely, a number of synthetic lethal screens have been performed in *Drosophila melanogaster* models to identify factors that restrain E2F–mediated apoptosis190,[191]. In these studies, the epidermal growth factor receptor (EGFR) and apoptosis inhibitor 5 (API5, also known as AAC11) were identified as required to suppress apoptosis arising from deregulated E2F activity. These studies suggest that EGFR antagonists may have significant utility in those tumours that harbour loss of RB function[191]. Furthermore, API5 is upregulated in a number of tumour cell types, and depletion of API5 specifically mediated cell death of tumour cells190. Thus, drugs targeting these ‘survival’ factors could be highly effective in RB-deficient tumours.

Although these screens clearly have the power to define genetic and functional interactions in model systems, it is equally important to decipher tumour-specific relationships that can be targeted therapeutically. Retinoblastoma is a tumour type where such relationships can be investigated against a high background of RB loss. Analyses of a combination of mouse models and human tumours suggested that inhibition of p53 function is a crucial determinant in the genesis of retinoblastoma[192] (FIG. 4). Furthermore, in human tumours this event was associated with the amplification of *MDM2* or *MDM4*; therefore, targeting MDM2 or MDM4 function may be of benefit in the treatment of retinoblastoma. Consistent with this concept, therapeutic agents that antagonize MDM2 and MDM4 function (for example, nutlins) show promise in murine models of retinoblastoma[192–]194. Additional studies in other tumours support the general concept that loss of RB may be particularly important in facilitating the cytotoxic effects of nutlin-3a195. Based on these findings, it is evident that tissue- and context-specific functions of RB provide a rich foundation for the development of future targeted therapeutic strategies.

Summary

Although RB was identified as the first *bona fide* tumour suppressor over 20 years ago, the implications of RB loss for cancer progression and tumour management are only now being uncovered. It is clear that RB serves as a gatekeeper of proliferative control, and that perturbations of RB function can occur through multiple tumour-specific alterations. These lesions arise in a non-overlapping fashion suggesting that they similarly perturb RB function; however, heterogeneity in the RB pathway is clearly relevant to both the aetiology of specific

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tumour types and disease behaviour. The specific import of RB deficiency is unmasked in the presence of cellular stress, including those introduced during cancer therapy. In hormone-dependent cancers, evidence suggests that RB depletion is sufficient to bypass endocrine-based therapeutic regimens. Similar results are observed with kinase inhibitors that antagonize distinct signalling pathways. Given the importance of these strategies in current and future tumour management, these findings may have significant clinical ramification. By contrast, RB deficiency can sensitize tumour cells to a subset of cytotoxic agents. Together, these findings identify RB as a crucial node that could be developed as a component of tailored anticancer strategies. However, formidable questions remain. First, what is the nature of tissue-specific and lesion-specific RB perturbation, and how do the differential mechanisms used to compromise RB function affect its predictive value? Second, how can RB deficiency be expediently identified in human tumour specimens? Third, can the predictive value of RB status be validated in prospective studies? At present, few clinical studies have considered RB status as a predictive factor for therapeutic response. Last, how can knowledge of RB function and cooperative factors be optimized so as to elicit maximal, tumour-specific cytotoxic response in RB-deficient contexts? Once addressed, it is hoped that these and similar queries will define the denouement of investigation of RB perturbations in human tumours, and will provide the foundation for translating decades of RB-related research to the clinic.

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Acknowledgments

The authors would like to thank all of their colleagues for thought-provoking discussions that provide the basis for the current Perspective. In particular the authors appreciate the contributions of W. Cavenee, R. Bremner, and T Tlsty to the preparation of the manuscript. A concerted effort was made to provide a highly inclusive discussion of the field: any omission was accidental and the authors apologize for not being able to cite all of the outstanding studies in the literature. The authors are supported by grants from the NIH: CA10617 and CA104213 to E.S.K. and CA099996 and CA116777 to K.E.K.

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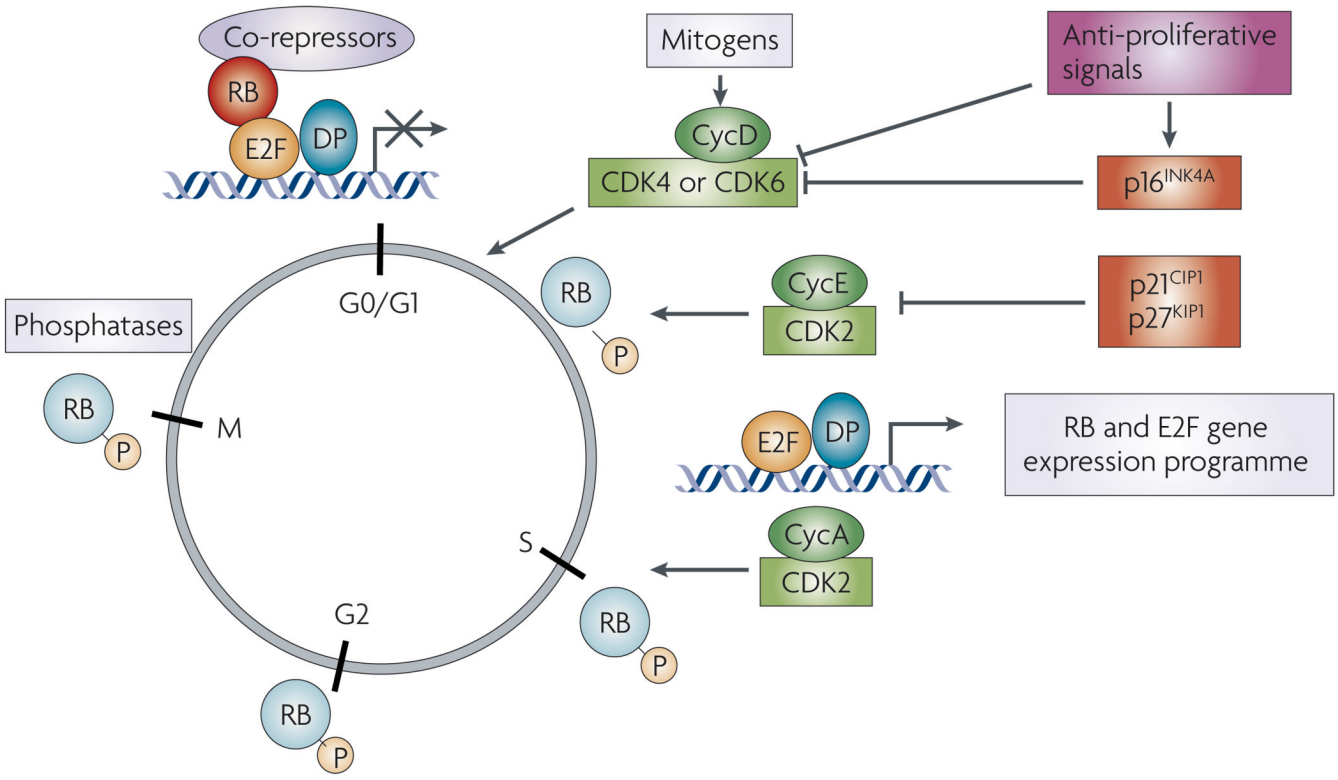

Figure 1. Schematic of RB in cell cycle control

Mitogenic signals stimulate the expression of D-type cyclins (Cyc) and a concomitant increase in cyclin-dependent kinase 4 (CDK4) and CDK6 activity. These factors initiate RB phosphorylation, which is augmented by the activity of CDK2 complexes with cyclins A and E. The phosphorylation of RB disrupts its association with E2F. This inactivation of RB allows for the expression of a transcriptional programme that enables progression through S-phase and mitosis. At the transition from mitosis to G1, RB is dephosphorylated through the action of phosphatases. Importantly, a large number of anti-mitogenic signals function to prevent RB phosphorylation either by limiting the activity of CDK4, CDK6 and CDK2 complexes or by inducing the activity of CDK inhibitors.

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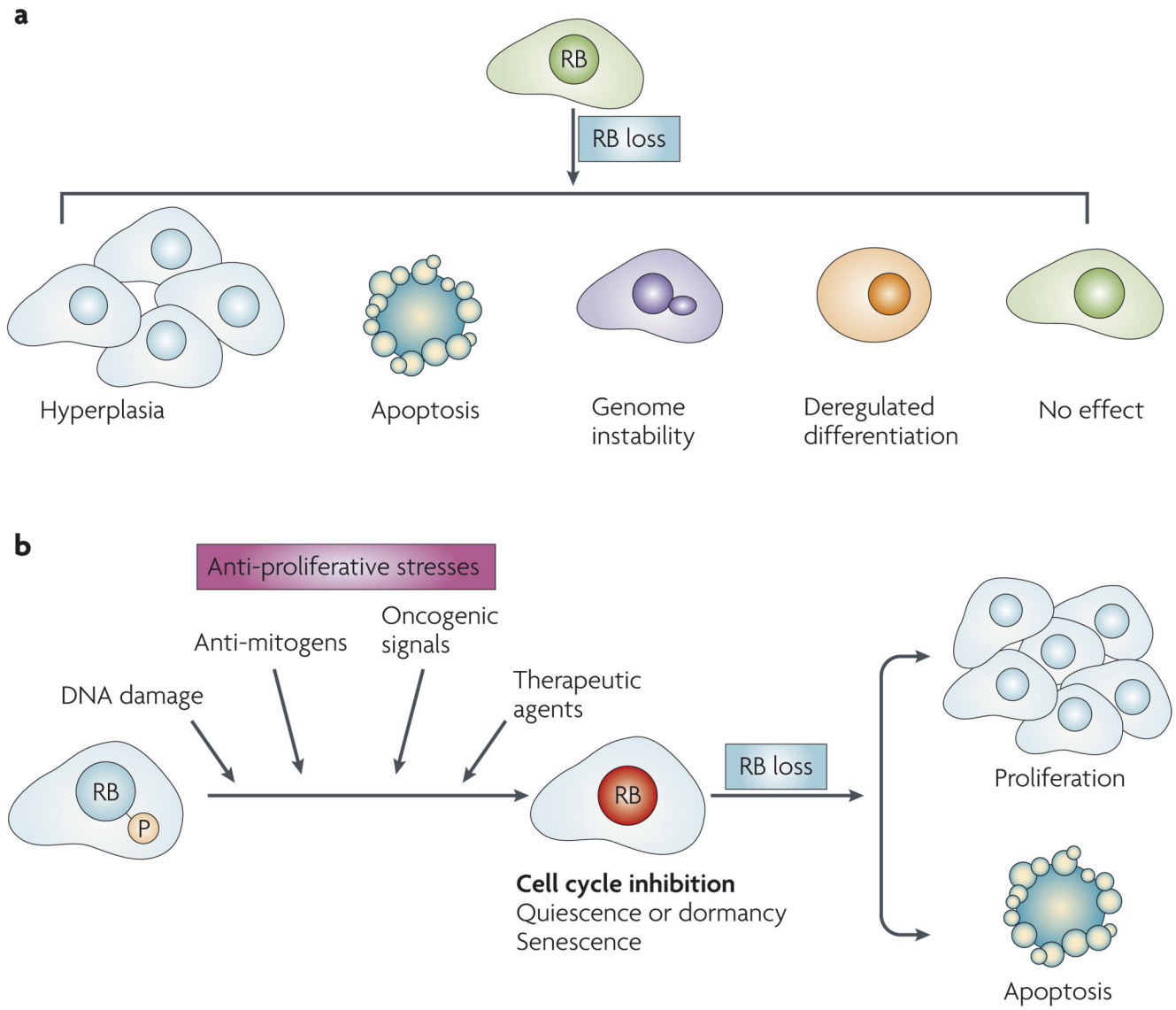

Figure 2. Influence of RB loss: context dependence

a | RB loss leads to diverse outcomes, such as hyperplasia or genome instability, in different cellular and tissue contexts. **b** | A diverse class of signals leads to the dephosphorylation and activation of RB. Activated RB can mediate cell-cycle inhibition leading to quiescence and/or dormancy or senescence. With the loss of RB, it is possible to escape cell-cycle inhibition and progress to either proliferation or apoptotic cell death.

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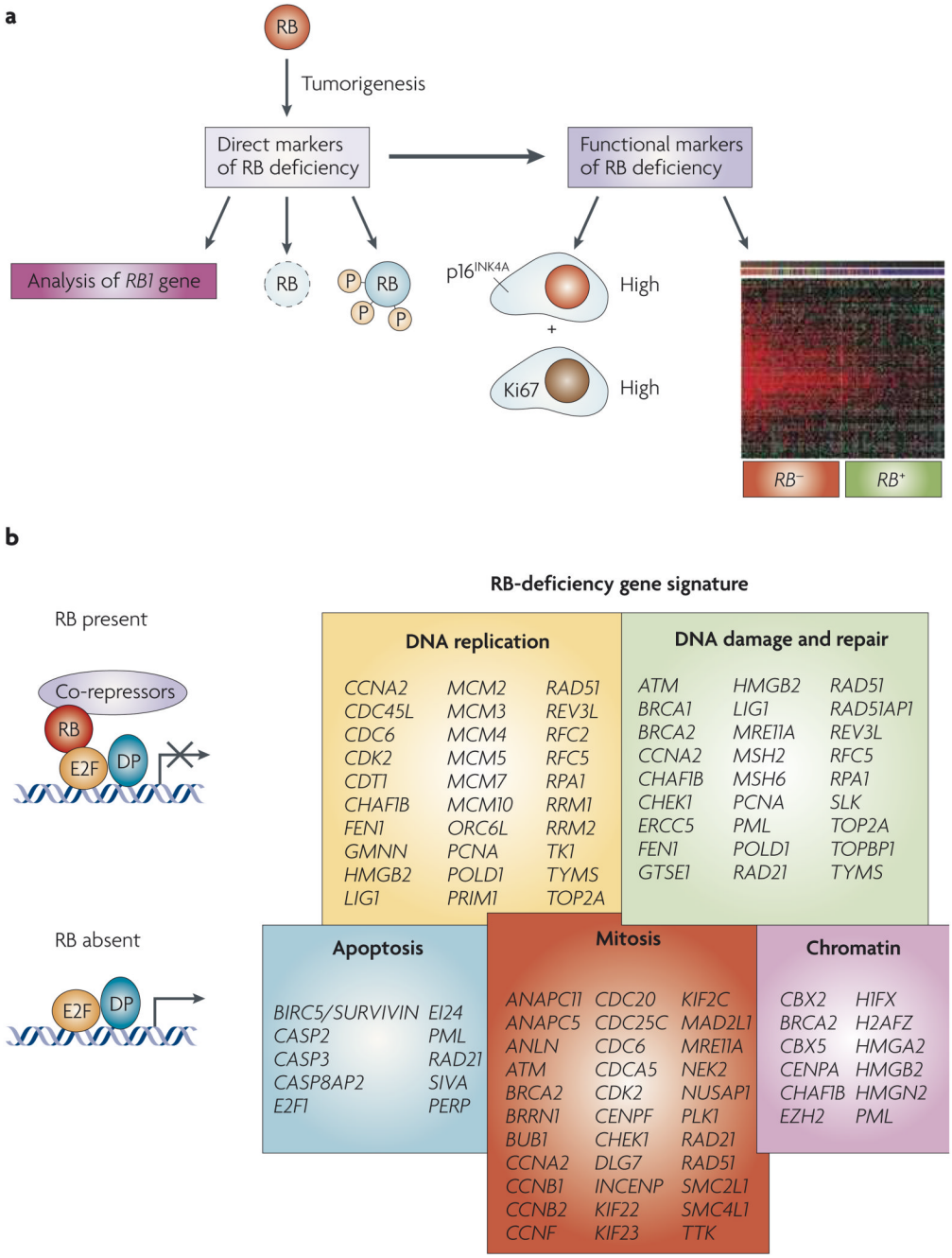

Figure 3. Mutiple markers for RB dysfunction

a | Direct analyses of the status of *RB1* are used in genetic tests to establish the presence of heritable retinoblastoma. RB levels can be directly interrogated by immunohistochemical analyses and the use of phospho-specific RB antibodies provides additional information as to the status of RB. However, as RB can be inactivated through multiple mechanisms, indirect markers for RB function can also be particularly informative. In this context, gene expression signatures reveal the downstream consequence of RB loss. $p16^{INK4A}$ levels are increased in specific RB-negative tumours, and these cells can be discerned from pockets of senescent pre-neo-plastic cells by the inclusion of a proliferative marker such as Ki67. **b** | Gene-signature analyses have shown the functional groups of genes that are deregulated by the loss of *RB1*

through either deletion or expression of viral oncoproteins. This gene expression signature overlaps with the gene expression grade index and the Oncotype Dx signatures by 65% and 80% respectively.

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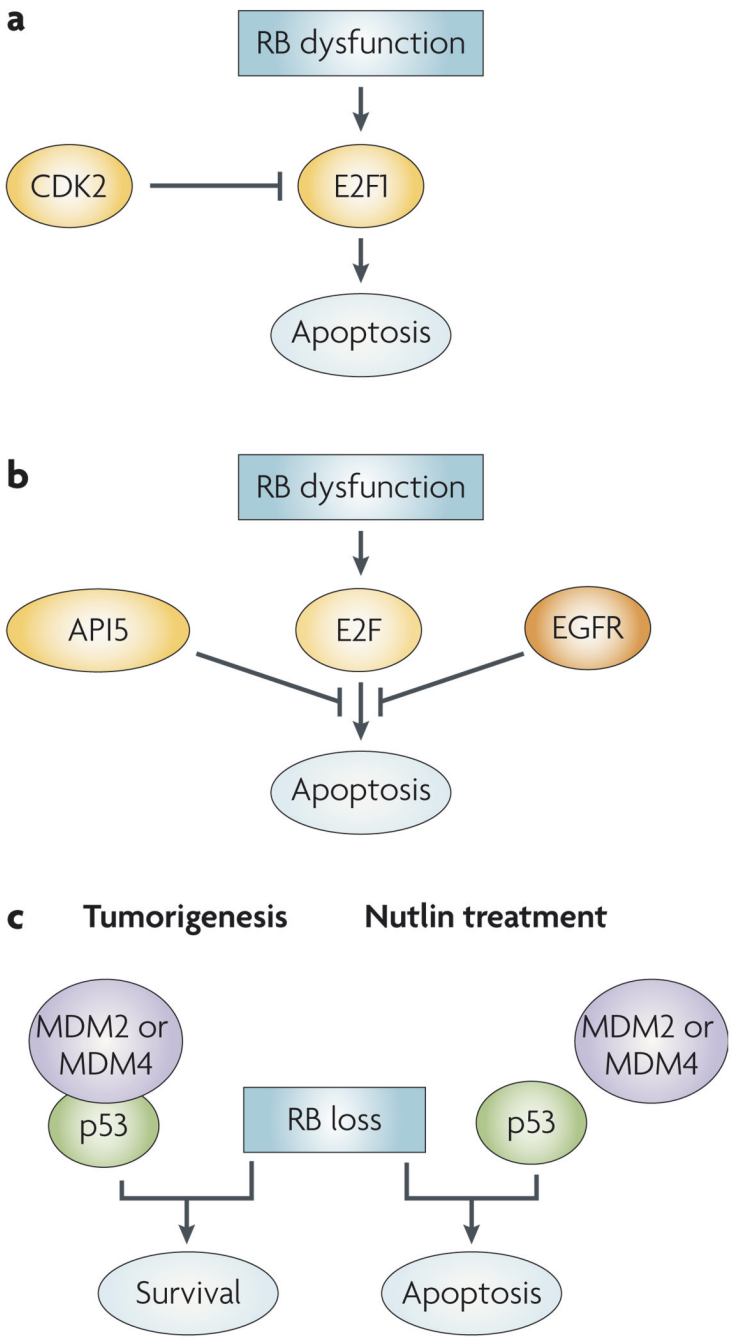

Figure 4. Exploiting RB deficiency therapeutically

a | RB limits the pro-apoptotic activity of E2F1, so RB-deficient cells are more prone to apoptosis. Cyclin-dependent kinase 2 (CDK2) activity further limits E2F1 activity. Thus, CDK2 inhibitors can further increase E2F1 activity and drive RB-deficient cells to apoptosis. **b** | In *Drosophila melanogaster* models, E2F activity can induce apoptosis; however, this is limited by the action of epidermal growth factor receptor (EGFR) signalling and the apoptosis inhibitor 5 (API5, also known as AAC11). **c** | In mouse models of retinoblastoma, p53 inactivation is required for tumour development and the survival of RB-deficient cells. In tumorigenesis, this inactivation occurs owing to an upregulation of MDM2 or MDM4. The

activity of these oncogenes can be targeted by the nutlins, a group of drugs that disrupt the interaction between MDM2 and p53.

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Table 1

RB pathway heterogeneity in primary tumours

Cancer type	Loss of $p16^{INK4A}$	Overexpression of cyclin D1*	Loss of RB	RB activity (indirect)	Refs
SCLC	<10% ND	<10% ND	>90% ND	Not assessed	54,59,196
NSCLC	40–60% indeterminate	40–60% indeterminate	10–20% indeterminate	Poor outcome	31,196–203
Bladder	20–40%poor outcome	20–80% favourable outcome	30–70% poor outcome	Not assessed	199,204_208
Prostate	10–40% indeterminate	0–35% indeterminate	30–60% indeterminate	Poor outcome	31,78,109, 111,144_148, 150, 209_213
Melanoma	40–60% poor outcome	30–60% no influence	10–40% no influence	Not assessed	214_219
Pancreas	40–70% poor outcome	50–70% indeterminate	<5% ND	Not assessed	220_225
Colorectal	30–60% no influence	40–60% no influence	0–20% no influence	Not assessed	203,226_228
Liver	30–70% indeterminate	20–40% indeterminate	20–40% indeterminate	Poor outcome	174,229_237
Breast	20–40% indeterminate	30–70% favourable outcome (ER-positive)	10–30% indeterminate (ER-negative)	Poor outcome	31,104,118, 127,168,174, 229_237

The percent of lesions in each pathway constituent is reflective of reports in the published literature and where possible represents a consensus range. The effect of the lesion in regard to survival is noted. In certain contexts a consensus based on tumours analysed has not been reached (indeterminate). Unless otherwise noted outcome is measured as survival.

*Functional RB status was determined by indirect measures in these analyses. ER, oestrogen receptor; ND, not determined; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

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Table 2

Preclinical assessment of RB loss and therapeutic response

Agent	Model	RB modulation	Outcome	Refs
Hormonal antagonism				
Oestrogen withdrawal	ER^+ breast cancer	Knockdown	Bypass	118
Tamoxifen	ER^+ breast cancer	Knockdown, viral oncoproteins	Bypass	118,119
ICI182780	ER^+ breast cancer	Knockdown, viral oncoproteins	Bypass	118
Androgen withdrawal	Prostate cancer	Knockdown, viral oncoproteins	Bypass	136,137, 143
Casodex	Prostate cancer	Knockdown	Bypass	143
Cytotoxic chemlotherapy				
Cisplatin	Breast cancer, lung cancer, murine fibroblasts, prostate cancer*	Knockout, knockdown	Sensitivity/ Resistance*	35,81, 118,151
5-Fluorouracil	Lung cancer, murine fibroblasts	Knockout, knockdown, viral oncoproteins	Sensitivity	16,151, 238,239
Ionizing radiation	Breast cancer, murine fibroblasts	Knockout, knockdown, viral oncoproteins	Sensitivity	81,118, 240
Doxorubicin	Lung cancer, murine fibroblasts	Knockout, knockdown, oncoproteins	Sensitivity	16,81, 82,151
Kinase inhibitors				
U0126	Murine fibroblasts, transformed murine fibroblasts	Knockout, spontaneous loss	Bypass	155
Staurosporine and UCN01	Murine fibroblasts, breast cancer, prostate cancer	Knockout, knockdown	Bypass	156_158
PD 332991	Breast cancer, lung cancer	Spontaneous loss	Bypass	159,160

Tumour lines or the use of murine fibroblasts are indicated. The model through which RB deficiency was produced is indicated (knockout reflects use of gene-targeted murine cells, knockdown reflects use of RNA-interference-based approach and viral oncoproteins reflect E1A or SV40 large T-antigen reagents that target pocket proteins). Bypass indicates a proliferative advantage with RB deficiency, whereas sensitivity indicates that RB deficiency decreased cellular viability.

*Resistance to cisplatin treatment in RB-deficient cells was observed in prostate cancer models. ER, oestrogen receptor.