COORDINATED REGULATION OF LIFE AND DEATH BY RB

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Recent studies have shown that RB can inhibit apoptosis, independently of its ability to block cell proliferation. This poses the question of how cells choose to grow or to die when RB becomes inactivated. RB is phosphorylated following mitogenic stimulation, but it is degraded in response to death stimuli. Most sporadic cancers also inactivate RB by phosphorylation, rather than losing RB entirely — possibly to exploit the survival advantage conferred by RB under stress. Drawing from the different mechanisms of RB inactivation, we propose two models for ways in which cells use RB to make the choice of life versus death.

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The retinoblastoma-susceptibility gene (*RB*) is a prototypical tumour suppressor. Germline mutation in the *RB* gene causes the highly penetrant hereditary retinoblastoma, which results from the bi-allelic loss of *RB* in embryonic retinoblasts. Consistent with its tumour-suppressor function, RB inhibits cell proliferation. The mechanism by which RB inhibits cell growth has been elucidated. Through its interaction with the E2F family of transcription factors, RB represses genes that are required for DNA synthesis[1]. RB-mediated repression of transcription occurs at two levels. First, RB blocks the interaction between E2F and other transcription co-activators[1]. Second, RB recruits chromatin-modifying enzymes to further repress transcription[1–3]. The inactivation of RB, therefore, is a prerequisite for cell proliferation.

RB is known to be inactivated by four mechanisms (FIG. 1). First, genetic inactivation of *RB* is observed in retinoblastoma, although the frequency of *RB* mutation is low among sporadic cancers. Second, viral oncoproteins, such as T antigen, E1A and E7, sequester RB from its physiological partners, thereby disrupting its function[1]. Third, RB can be inactivated by phosphorylation, which is catalysed by the cyclin-dependent protein kinases (CDKs) during cell-cycle progression. Finally, RB is degraded by caspases in response to apoptotic stimuli. In all cases, the inactivation of RB can confer a proliferative advantage, due to the derepression of E2F-regulated genes[1]. Interestingly, *RB* mutation, RB sequestration by viral proteins and RB degradation by caspases have each been associated with increased levels of apoptosis. Recent studies in mice have shed new light on the mechanisms by which RB regulates cell death, and indicate its direct role in inhibiting apoptosis.

Apoptosis in *Rb*-null mice

Studies with *Rb*-null mice more than a decade ago first revealed the role of Rb in apoptosis regulation. Phenotypes of these mice include excessive apoptosis of cells in the nervous systems, lens and skeletal muscles[4–6]. Breeding of *Rb*-heterozygous mice with other genetically engineered strains has identified suppressors of the ectopic apoptosis phenotype, indicating that these gene products interact with Rb in apoptosis regulation (TABLE 1; FIG. 2). Mutation of the mouse p53 gene (*Trp53*$^{-/-}$) diminished apoptosis in the lens and the central nervous system (CNS) of *Rb*-null embryos[7,8], and mutation of *E2f1* also suppressed apoptosis of the *Rb*-null CNS neurons and the lens epithelial cells[9,10]. A common target gene for transcriptional regulation by E2f1 and p53 is apoptosis protease activating factor-1 (Apaf1). Apaf1 is the key component of the apoptosome — a downstream effector of mitochondria-dependent apoptosis[11]. E2F1 and p53 binding sites have been mapped in the promoter of *Apaf1* (REF. 12). CNS neurons of *Apaf1*-null embryos are resistant to apoptosis induced by adenovirus-expressing p53 (REF. 13). Consistent with these observations, *Apaf1* expression is

Summary

- Loss of RB sensitizes cells to apoptosis.
- Ectopic apoptosis of *Rb*-null neurons is not a default outcome of inappropriate S-phase entry.
- RB can be inactivated by phosphorylation and degradation.
- RB degradation is required for tumour necrosis factor type I receptor-induced apoptosis.
- Most sporadic human cancers inactivate RB function by exploiting pathways that regulate RB phosphorylation.
- Loss of RB can only contribute to tumour development under conditions in which apoptosis response is compromised.

deregulated in *Rb*-null CNS neurons[12], and the pro-apoptotic phenotype of *Rb*-null CNS is suppressed by the mutation of *Apaf1* (REF. 14). Therefore, in the absence of Rb, deregulated E2F1 and p53 contribute to the activation of mitochondria-dependent apoptosis in the CNS (FIG. 2).

Interestingly, elimination of *E2f1*, *Trp53* or *Apaf1* did not reverse the apoptotic phenotype of *Rb*-null neurons in the peripheral nervous system (PNS)[7,9,14]. However, mutation of the related transcription factor, *E2f3*, did suppress PNS apoptosis in *Rb*-null embryos[10,15]. In cultured embryonic fibroblasts, deletion of *E2f1*, but not *E2f3*, prevented Myc-induced apoptosis[16]. Nevertheless, heterozygous or homozygous deletion of *E2f3* protected the *Rb*-null CNS and PNS neurons from apoptosis[10,15]. So, the deregulation of E2f3 in *Rb*-null neurons is an important contributing factor to apoptosis. These observations also indicate that the transcription function of E2f1 and E2f3 might be programmed differently in developing neurons and cultured fibroblasts.

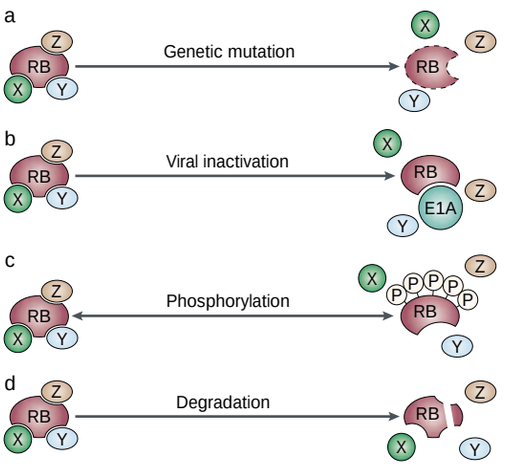


Figure 1 | **Mechanisms of RB inactivation.** Retinoblastoma (RB) contains multiple protein binding sites and functions as a molecular scaffold to promote the assembly of transcription complexes. Disassembly of these complexes is mediated by RB inactivation by means of four known mechanisms. **a** | The *RB* gene is mutated (dashed line), causing release of its associated factors. *RB* mutations have been detected in retinoblastoma and a small fraction of sporadic tumours. **b** | RB is sequestered by viral oncoproteins, such as E1A, which prevent it from binding other factors. **c** | Phosphorylation (P) of RB by CDK–cyclin complexes during cell-cycle progression disrupts its ability to assemble transcriptional complexes. **d** | RB is degraded by a caspase-dependent proteolytic pathway during apoptosis. Except for RB phosphorylation (**c**), the other three mechanisms of RB inactivation (**a**, **b** and **d**) have been associated with sensitization to apoptosis.

Apoptosis of the *Rb*-null PNS neurons can also be suppressed by mutation of the caspase-3 gene (*Casp3*)[17]. In the apoptosis signalling pathway, Apaf1 functions upstream of Casp3 (REF. 11). In the developing nervous systems of *Rb*-null embryos, *Apaf1*-knockout rescued apoptosis in the CNS, and *Casp3*-knockout rescued apoptosis in the PNS[14,17]. These observations have several implications. First, death of *Rb*-null neurons requires the apoptotic machinery, because it can be prevented by the loss of *Apaf1* or *Casp3*. Second, in the absence of Rb, CNS neurons undergo apoptosis through E2f1- and p53-dependent transcription of *Apaf1*. The upregulation of *Apaf1* is necessary, but probably not sufficient, to kill cells, as external stress signals might also be required. Third, apoptosis of *Rb*-null CNS neurons can occur in the absence of Casp3 (REF. 17), so other caspases might be involved in killing CNS neurons.

Mutation of a single caspase in mice has been shown to cause compensatory activation of other caspases[18] — the observed death of $Rb^{-/-}Casp3^{-/-}$ CNS neurons could be the result of such adaptation. Recent studies have identified E2F-binding sites in the promoters of several caspase genes, including *Casp3* (REF. 19). In the PNS of *Rb*-null embryos, deregulation of E2F3 might lead to increased *Casp3* expression, and therefore sensitize these neurons to apoptosis (FIG. 2).

Apoptosis of the *Rb*-null skeletal muscles was observed in neonatal mice of two different genetic backgrounds[20,21]. One group of mice was created by disruption of both the *Rb* and *Id2* genes[20]. Id2 is a helix–loop–helix transcription regulator that can bind to Rb[22]. Mutation of *Id2* rescued most of the defects of *Rb*-null embryos[20]. Nevertheless, these mice died at birth with a severe reduction of muscle mass[20]. It is not yet clear how *Id2* deficiency rescues the *Rb*-null embryonic lethality. However, it is clear that *Id2* knockout does not suppress the death of *Rb*-null muscles.

The second strain of mice was created through the transgenic expression of a wild-type *Rb* minigene (which consists of a genomic fragment that spans 1.3 kb of the mouse *Rb* promoter, the first exon and the intron fused to exons 2 to 27 of the mouse *Rb* cDNA) in *Rb*-null embryos[23]. The *Rb* minigene was expressed in neurons but not in muscle cells, and rescued the ectopic neuronal apoptotic phenotype, but skeletal muscles of these mice still underwent apoptosis[21]. Mutation of *Trp53* or *E2f1* did not rescue the muscle apoptotic phenotype in these mice[21]. Mutation of *Cdkn1a* (which encodes Cip1, also known as p21), a Cdk inhibitor, increased levels of apoptosis in *Rb*-null muscle cells[21]. Taken together, observations made in the various strains of *Rb*-null mice indicate that Rb can suppress apoptosis induced by different pathways during development (FIG. 2).

Table 1 | Summary of *Rb*-mutant mouse strains

Genotype	Lethality	Tumour spectrum	Major phenotypes	References
Rb$^{-/-}$	E13.5	ND	Increased ectopic S-phase entry and massive apoptosis in neurons; defects in terminal differentiation of muscle and erythrocytes	4,5,6
Rb$^{+/-}$	Shortens lifespan	Pituitary	ND	59
Rb$^{+/-}$ *Trp53*$^{-/-}$	Shortens lifespan	Pituitary, thyroid pancreatic islet, soft-tissue sarcoma	*Trp53* dosage-dependent survival (lifespan shortens as *Trp53* gene dosage decreases)	62,63
Rb$^{+/-}$ *E2f1*$^{-/-}$	Lengthens lifespan compared to that of *Rb*$^{+/-}$	Pituitary, lung, adenocarcinoma, thyroid	Decreased incidence of pituitary tumours, but increases the spectrum of tumours (similar to the kinds of tumour that develop in *E2f1*$^{-/-}$ mice)	64
Rb$^{-/-}$ *Trp53*$^{-/-}$	E13.5	ND	Reduced apoptosis in CNS but not in PNS; increased ectopic S-phase entry is observed in both CNS and PNS	7,8
Rb$^{-/-}$ *E2f1*$^{-/-}$	E17	ND	Reduced ectopic S-phase entry and apoptosis in CNS and lens tissue; upregulation of p53 activity was abolished in *Rb*$^{-/-}$ brains; skeletal muscle mass and the number of muscle fibres were significantly reduced; defects of lung development were observed	9
Rb$^{-/-}$ *E2f2*$^{-/-}$	E13.5	ND	Partial reduction of ectopic S-phase entry in both CNS and lens tissue; no reduction of apoptosis in these tissues was observed	10
Rb$^{-/-}$ *E2f3*$^{-/-}$	E17.5	ND	Reduced ectopic S-phase entry and apoptosis in CNS, PNS and lens tissue; two classes of phenotype were observed in *Rb*$^{-/-}$ *E2f3*$^{+/-}$ embryos (one class showed complete suppression of both ectopic entry and apoptosis in CNS, whereas the other class showed only suppression of apoptosis)	10,15
Rb$^{-/-}$ *Id2*$^{-/-}$	At birth	ND	Embryos survived to term; normal neurogenesis and haematopoesis; normal apoptosis in CNS but moderate increase of apoptosis in PNS (however, mice died at birth due to severe defects in muscle differentiation — greatly reduced number of muscle fibres)	20
Rb$^{-/-}$ *Apaf1*$^{-/-}$	E13.5	ND	Complete suppression of apoptosis in CNS and lens tissue; partial reduction of apoptosis in PNS	14
Rb$^{-/-}$ *Casp3*$^{-/-}$	E13.5	ND	Rescue of apoptosis in PNS (trigeminal ganglia, dorsal root ganglia); *Casp3* deficiency did not rescue the CNS neurons from apoptosis in *Rb*-null embryos	17
Rb$^{-/-}$ (telencephalon)	Die minutes after birth (respiratory defects)	ND	No suppression of ectopic S-phase entry or mitosis was observed in *Rb*-null cells; the number of tunel-positive cells was suppressed compared to *Rb*-null embryos; *Rb*-null cells in telencephalon area express early neuronal markers; slight increase in telencephalon area was observed, indicating enhanced neurogenesis	25
Phosphor-ylation-resistant *Rb* (mammary gland)	Normal lifespan	Mammary adenocarcinoma	Suppression of ductal growth in pubescent stage (5–7 weeks of age); development of multiple suppression of hyperplastic nodules in the mammary gland; apoptosis during postlactational involution	61
Rb$^{MI/MI}$	Normal lifespan	None	Protection of male mice from lipopolysaccharide-induced mortality; MI cells are resistant to apoptosis induced by type I TNFR but remain sensitive to DNA-damage-induced apoptosis	29

CNS, central nervous system; ND, not detected; None, no tumour found; PNS, peripheral nervous system.

Pros and cons of the 'conflict' model

The apoptosis phenotype of *Rb*-null embryos is correlated with the ectopic S-phase entry of *Rb*-null neurons and muscles[4–6,24]. This correlation has led to the hypothesis that apoptosis results from a 'conflict' in the *Rb*-null progenitor cells (FIG. 3a). The conflict model is based on the ability of RB to block cell-cycle progression and to facilitate differentiation. In the absence of RB, cells cannot cease proliferation despite the stimulation by differentiation signals. Continuation of DNA synthesis during differentiation results in a conflict that triggers apoptosis. This model

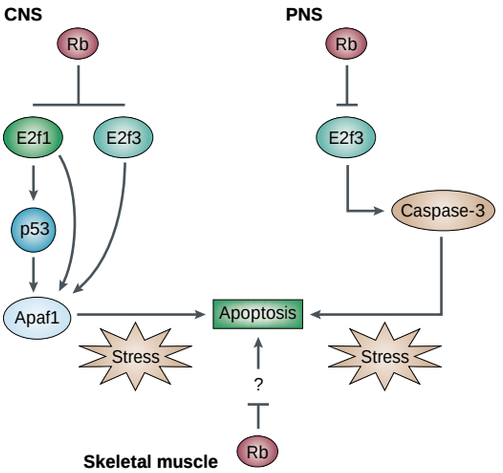


Figure 2 | **Genetic inactivation of Rb sensitizes developing neurons and skeletal muscle to apoptosis.** The *Rb*$^{-/-}$ mouse embryos undergo excessive apoptosis in the central nervous system (CNS), peripheral nervous system (PNS) and skeletal muscle. Neuron-specific disruption of *Rb* does not lead to apoptosis[25], so additional factors are required to induce apoptosis of neurons of *Rb*-null embryos. In the CNS of *Rb*-null embryos, disruption of the genes *E2f1* (REF. 9), *E2f3* (REF. 15), *Trp53* (REF. 8) or *Apaf1* (REF. 14) can suppress excessive apoptosis. The *Apaf1* gene is regulated by E2f1 and p53 (REF. 12). In the PNS of *Rb*-null embryos, loss of E2f3 (REF. 15) or caspase-3 (REF. 17), but not E2f1, p53 or Apaf1, prevents excessive apoptosis. The *Casp3* gene is regulated by E2f[19]. The excessive apoptosis that occurs in *Rb*-null skeletal muscle is not suppressed by *E2f1* or *Trp53* gene disruption[21]. These results indicate that Rb can suppress apoptosis that is induced by several pathways during mouse development.

proposes that RB does not directly protect progenitor cells from apoptosis. Instead, the increased apoptosis of *Rb*-null cells is the default outcome of ectopic S phase.

Recent results from conditional *Rb*-knockout mice have shown that, in the developing CNS, ectopic S-phase entry does not lead to apoptosis by default[25,26]. By combining a conditional *Rb* knockout with neuron-specific *Rb* expression, Slack and colleagues targeted the inactivation of *Rb* in the developing telencephalon[25]. These mice survived through term and were born with the expected Mendelian ratio. However, they died within 20 minutes of birth, due to unexplained respiratory defects. The *Rb*-inactivated neurons showed ectopic S-phase entry and mitosis, but the inappropriate proliferation did not lead to apoptosis[25]. Jacks and colleagues have made a similar observation with an independently derived conditional knockout of *Rb*[26]. At present, it is not understood why the general knockout of *Rb* causes neurons to undergo apoptosis. It has been proposed that hypoxic stress, possibly caused by the developmental defects of *Rb*-null red blood cells, might contribute to the apoptosis of *Rb*-null neurons[26]. In a modified 'conflict' model, ectopic proliferation, combined with stress generated in the *Rb*-null background, causes *Rb*-null progenitor cells to undergo apoptosis (FIG. 3b).

An alternative model proposes that Rb directly inhibits stress-induced apoptosis (FIG. 3b). This model proposes that Rb has an anti-apoptotic function in addition to its well-established antiproliferative function. Recent experiments have added credence to the idea that Rb directly inhibits apoptosis. The ability of Rb to repress E2F1-dependent gene expression and the ability of E2F1 to induce apoptosis are well established[1,16]. Thymocytes from *E2f1*-null mice are resistant to activation-induced apoptosis[27]. Fibroblasts from *E2f1*-null mice are resistant to Myc-induced apoptosis[16]. Helin and colleagues have identified a number of genes that are upregulated by a conditionally activated E2F1 (REF. 28). As expected, E2F1 stimulated the expression of cell-cycle genes — for example, the G1/S cyclins. Interestingly, E2F1 also induced the expression of genes that encode the cell-death machinery, including several caspases and Apaf1 (REF. 28). E2F-binding sites have been defined in the *Apaf1* promoter[12]. Recently, *in silico* analysis of the human genome has identified E2F-binding sites in promoters of several caspase genes[19]. These results support the conclusion that E2F1 is a *bona fide* pro-apoptotic transcription factor. The fact that RB can repress transcription from E2F1-occupied promoters makes it a negative regulator of apoptosis.

RB attenuates apoptotic signalling

The ability of Rb to directly inhibit apoptosis is also supported by the phenotype of a strain of mice that express a caspase-resistant Rb[29]. As discussed above, RB can be degraded through the action of caspase[30]. Caspases function in transducing death signals and executing cell killing. Since *Rb*-null cells are hypersensitive to apoptosis induced by a variety of agents, Rb degradation could contribute to cell death. RB contains several consensus sites for caspase cleavage[30–32]. The main caspase cleavage site in RB is located at the extreme carboxyl terminus. Mutation of this carboxy-terminal cleavage site creates a degradation-resistant form of RB, called RB-MI (mutated at the ICE-site)[33]. The ectopic expression of RB-MI attenuated apoptosis that was induced by tumour necrosis factor-α (TNF-α) in fibroblasts[33] and by potassium deprivation in cultured cerebellar granular neurons[34]. These results indicate that RB is an important substrate of caspase, and that RB degradation contributes to the induction of apoptosis.

To investigate the physiological significance of RB degradation in apoptosis, our laboratory has replaced the wild-type *Rb* allele with the *Rb-MI* allele in the mouse germline[29]. The *Rb-MI* mice are healthy and fertile with a normal lifespan. When challenged with bacterial endotoxin, the *Rb-MI* mice showed tissue-specific resistance to apoptosis[29]. In cells of the intestine, the presence of a single *Rb-MI* allele was sufficient to protect against apoptosis, showing that preservation of Rb can inhibit cell death. On the other hand, splenocytes of heterozygous or homozygous *Rb-MI* mice were not protected from apoptosis during endotoxic shock[29].

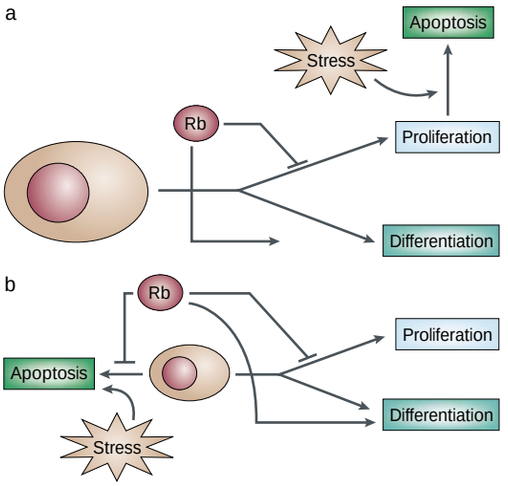


Figure 3 | **Two explanations for the apoptosis phenotype of *Rb*-null cells.** During development, progenitor cells can choose among three fates: proliferation, differentiation or apoptosis. **a** | The 'conflict' model proposes that apoptosis is the default fate as *Rb*-null cells undergo unscheduled proliferation — either in the presence or absence of cellular stress. The conflict model considers suppression of apoptosis to be a secondary effect to the antiproliferative function of Rb. **b** | The alternative model proposes that suppression of apoptosis is a primary function of Rb, independent of its antiproliferative activity. Rb also promotes differentiation in both these models.

The tissue-specific protection could be explained by the finding that Rb-MI is resistant to degradation induced by the type I receptor for tumour necrosis factor (TNFRI)[29], but activation of TNFRI plus TNFRII signalling causes Rb-MI degradation[29]. TNFRI is widely expressed, whereas the type II receptor is preferentially expressed in the haematopoietic lineage, which would explain why splenocytes were not protected from apoptosis by *Rb-MI* expression. It will be of interest to construct a non-degradable form of RB, by mutating all of its caspase cleavage sites, to see whether it could protect cells from both TNFRI- and TNFRII-induced degradation. So, preservation of Rb protein, in the form of Rb-MI, can interfere with TNFRI-induced apoptosis and can exert tissue-specific protection of apoptosis *in vivo* — these results further support the concept that RB inhibits apoptosis.

But how does RB-MI function? First of all, Rb-MI protects against TNF-α-induced apoptosis through a mechanism other than suppressing cell-cycle progression. When treated with TNF-α and cycloheximide to induce apoptosis, DNA synthesis was equally inhibited in both *Rb-WT* and *Rb-MI* fibroblasts. Whereas both cells types arrested in G1, *Rb-WT* fibroblasts underwent apoptosis and *Rb-MI* cells remained viable. Further experiments with *Rb-MI* fibroblasts showed that this protein interferes with cytochrome *c* release and caspase-3 activation to protect against apoptosis[29]. This observation indicates that degradation of RB, through caspase, contributes to further activation of caspase. So, elimination of RB is required for caspase to transduce and/or amplify the apoptotic signal from TNF-α (BOX 1).

The concept that caspases cleave specific substrates to amplify the apoptotic signal is supported by recent findings that cleavage of RIP (receptor interacting protein, a dead domain serine/threonine kinase) and IKKβ (IκB kinase-β) can each contribute to TNF-α-induced apoptosis[35,36]. Preservation of RIP or IKKβ, through expression of non-cleavable variants of each protein, can protect cells from apoptosis by maintaining the activity of nuclear factor of κB (NF-κB)[35,36].

It is interesting to note that RB-MI did not interfere with cytochrome *c* release or caspase-3 activation when these events were induced by DNA damage[29]. Despite the presence of intact RB-MI, DNA-damage signals induced apoptosis without any detectable delay[29]. The signal-specific protection from apoptosis argues that RB-MI does not function as a general inhibitor of caspases. Instead, these results show that the transduction and/or amplification of apoptotic signals from DNA damage can proceed without RB degradation. DNA damage preferentially kills proliferating cells, which inactivate RB by phosphorylation. As RB-MI can be phosphorylated, its presence might be inconsequential in DNA-damage-induced apoptosis. As discussed below, either phosphorylation or degradation of RB might sensitize cells to apoptosis (FIG. 4). Alternatively, DNA damage and TNF-α might induce cytochrome *c* release and caspase-3 activation through distinct mechanisms, whereas RB-MI specifically inhibits the TNF-α-induced death pathway.

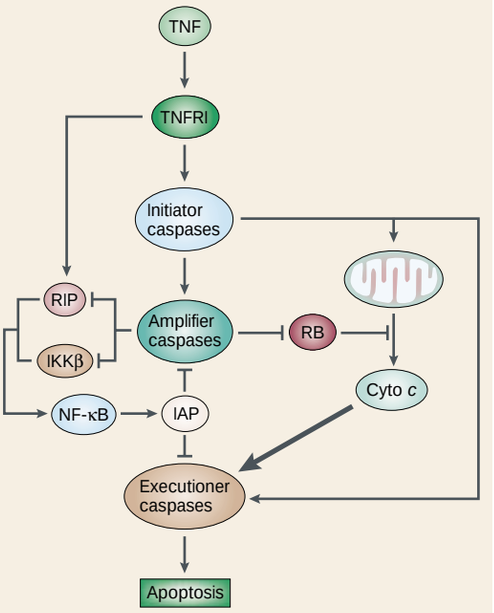
RB inhibits pro-apoptotic factors other than E2F1

As summarized in TABLE 1 and FIG. 2, apoptosis in *Rb*-null embryos is not always suppressed by *E2f1* gene disruption. This might be explained by the fact that E2F1 is only one of a hundred or so cellular proteins that have been shown to interact with RB[37]. Several of the reported RB-binding proteins have a pro-apoptotic function. The nuclear c-ABL tyrosine kinase can induce apoptosis[38], and RB directly binds to it and inhibits its kinase activity[39]. The JNK kinase is involved in stress-induced apoptosis, and this function seems to be inhibited by RB[40]. The amino-terminal region of RB contains a binding site for p84N5, which, when overproduced, has pro-apoptotic activity that is inhibited by RB[41,42]. It was reported that the large subunit of replication factor C can promote survival following DNA damage, and that this protection is dependent on its interaction with RB[43]. Taken together, these findings support the concept that RB regulates the activities of several proteins, including those that promote apoptosis.

The inhibition of E2f1 by Rb contributes to the repression of *Apaf1* and *Casp3* expression, and therefore suppresses apoptosis during mouse embryonic development. The regulated expression of *Apaf1* and *Casp3*, however, is not likely to fully control the apoptotic response to TNF-α. In fact, TNF-α-induced apoptosis is enhanced by cycloheximide and actinomycin D, which inhibit gene expression. This raises the interesting possibility that RB could inhibit apoptosis through mechanisms other than the regulation of gene expression. It is conceivable that RB participates in the

Box 1 | Caspase-mediated amplification of apoptotic signal

Tumor necrosis factor (TNF) is a cytokine that stimulates inflammation and apoptosis. Previous work has established the function of two types of caspases in TNF-induced apoptosis. The 'initiator' caspases — for example, caspase-8 — are directly activated by the type I TNF receptor (TNFRI). The 'executioner' caspases — for example, caspase-3 — are activated by the initiator caspases through mitochondria-dependent and -independent pathways, such as through cytochrome *c* (Cyto *c*) release. The previous model of direct connections between the initiator and executioner caspases would predict a rapid apoptosis following TNFRI activation. However, a rapid death would be incompatible with the physiological function of TNF, which is to regulate the inflammatory response to pathogens and stress. Recently, we have found that the cleavage of RB is required for TNFRI to activate the executioner caspases — that is, caspase-3. The nuclear location of RB and the timing of its degradation indicate that RB is cleaved by caspases that are in-between the initiators and the executioners. We therefore propose the term 'amplifier' to describe the caspase that cleaves RB. The amplifier caspases are not defined by their molecular identities, but rather by their function in apoptotic signal transduction. For example, caspase-3, which cleaves RB[29] and IκB kinase-β (IKKβ)[36], can function as an amplifier and as an executioner. The caspase-resistant form of RB, which is called 'RB-MI', can interfere with cytochrome *c* release from the mitochondria to diminish the processing of caspase-3 (REF. 29). The amplifier caspases might also cleave the pro-apoptotic proteins receptor interacting protein (RIP)[35] and IKKβ[36]. TNF activates nuclear factor of κB (NF-κB) through RIP and IKKβ to stimulate inflammation. NF-κB also inhibits apoptosis through the upregulation of IAPs that block caspases. By cleaving RIP and IKKβ, the amplifier caspases can extinguish inflammation and promote apoptosis. Caspase-resistant RB, RIP or IKKβ could each interfere with the function of the amplifier caspases to diminish the apoptosis response to TNF.



nuclear sequestration of factors that stimulate cytochrome *c* release. Caspase-mediated degradation of RB could release such factors to the cytoplasm, leading to the transduction and/or amplification of apoptotic signals.

To grow or to die?

Because the inactivation of RB can stimulate proliferation and apoptosis, and because the RB/E2F complex regulates the expression of S phase and apoptotic genes, the question arises as to how the decision to proliferate or to die is controlled. In response to mitogenic signals, cells inactivate RB by phosphorylation. Phosphorylation of RB is required for mitogens to stimulate DNA synthesis. In response to death-receptor signals, cells inactivate RB by caspase-dependent degradation. Degradation of RB, as we have shown, is required for TNFRI to induce apoptosis[29]. Because mitogenic and apoptotic signals inactivate RB through different molecular mechanisms, it is conceivable that RB/E2F promoter complexes could be differentially regulated.

This model of 'promoter-specific' regulation of RB/E2F transcription complexes is illustrated in FIG. 4a. The RB/E2F complex at the promoters of S-phase genes is regulated through RB phosphorylation. The RB/E2F complex at the promoters of apoptosis genes, on the other hand, is regulated through RB degradation. This model would predict that a fraction of the RB/E2F repression complexes are stable and functional in proliferating cells. Indeed, RB/E2F complexes have been detected in nuclear extracts from S-phase cells[44]. It remains to be determined whether these S-phase RB/E2F complexes occupy promoters of apoptosis genes. This model would also predict that RB/E2F complexes at some promoters to be resistant to CDK-mediated phosphorylation. Whether or how such phosphorylation-resistant RB complexes are assembled would be an interesting problem to solve.

An alternative model, illustrated in FIG. 4b as the 'context-dependent' regulation, can also explain how cells choose to proliferate or to die following RB inactivation. In this model, the different mechanisms of RB inactivation do not determine the cell fate. Rather, the activities of parallel pathways, in combination with RB inactivation, determine the biological outcome. FIGURE 4b shows a scenario in which mitogenic stimulation activates pro-survival factors, such as the transcription factor NF-κB[45] and the protein kinase AKT[46]. These survival signals antagonize the induction of pro-apoptotic genes caused by the inactivation of RB, thereby ensuring cell proliferation instead of apoptosis.

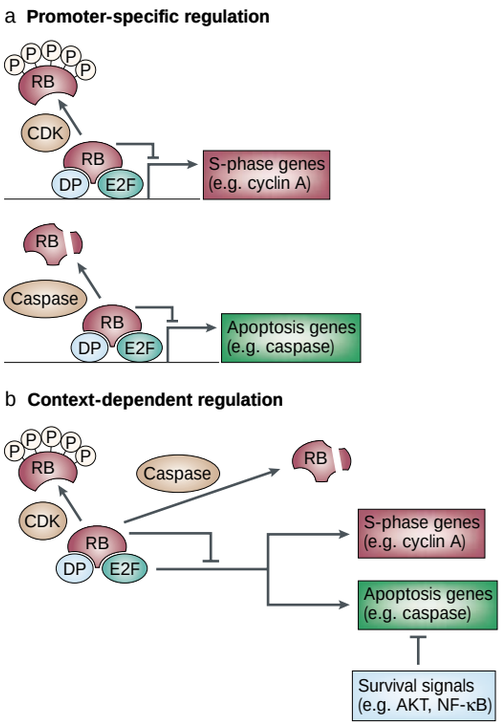


Figure 4 | **Two models for the differential regulation of proliferation versus apoptosis. a** | The 'promoter-specific regulation' model proposes that RB phosphorylation (P) by cyclin-dependent kinase (CDK) releases RB from the promoters of S-phase genes, allowing E2F (composed of E2F and DP heterodimer) to stimulate their expression. Phosphorylation does not disrupt the RB/E2F complexes that are assembled at the promoters of apoptosis genes. Instead, RB degradation, through the action of caspase, is required to disrupt the transcription repression complexes at the promoters of pro-apoptosis genes. **b** | The 'context-dependent regulation' model proposes that RB phosphorylation or degradation is equally effective at disrupting RB-mediated transcriptional repression of S-phase genes and pro-apoptosis genes. Following mitogenic stimulation and RB inactivation, cells do not undergo apoptosis because the expression of pro-apoptosis genes is counteracted by the activation of survival factors such as AKT and NF-κB. These two models are not mutually exclusive. In other words, promoter-specific regulation could be used in combination with parallel survival factors to control the decision of proliferation versus apoptosis.

Many studies have shown the importance of survival signalling in cell proliferation, supporting this model. The coordinated induction of proliferative and apoptotic genetic programmes after RB inactivation would also explain the preferential sensitization of proliferating cells to apoptosis. This model would predict that caspase-dependent inactivation of RB could contribute to cell proliferation if sufficient survival signals existed to antagonize the death function of caspases. The two models depicted in FIG. 4 are not mutually exclusive and could be used simultaneously to integrate the myriad of signals that affect the decision of life and death.

RB is a conditional tumour suppressor

The direct regulation of apoptosis by RB has important implications for tumour development. Because RB has a central role in the inhibition of cell proliferation, tumour development involves the inactivation of RB. However, as RB also has a crucial role in the inhibition of apoptosis, developing tumours need to preserve RB to antagonize apoptotic signalling. Previous work has shown that the 'RB pathway' is targeted for inactivation in more than 80% of sporadic human cancers[47]. Interestingly, the *RB* gene itself is rarely mutated in sporadic human cancers. Rather, tumour cells target the pathway that regulates the phosphorylation of RB. In particular, sporadic cancer cells repress the expression of *CDKN2A* (which encodes INK4A, also known as p16) which inhibits CDK4/6 (REF. 48) through gene deletion or promoter hypermethylation[49–51]. The increased expression of D-type cyclins that activate CDK4/6 is another strategy developed by tumour cells to inactivate RB. Cyclin D1 overproduction is observed in human mammary carcinomas[52], and cyclin-D1-null mice are resistant to mammary tumour development induced by the murine mammary tumour virus[53]. Activated CDK4/6–cyclin-D complexes not only phosphorylate and inactivate RB, but also phosphorylate and inactivate the RB-related proteins p107 and p130, which share redundant function with RB in the regulation of E2Fs[44]. Moreover, this phosphorylation is reversible. So, targeting the phosphorylation pathway of RB provides tumour cells not only with a growth advantage, but also with the option to re-activate RB for protection against apoptotic stimuli.

In keeping with the idea that RB inactivation needs to be coupled with apoptosis defects during tumour development, DNA tumour viruses have developed oncoproteins that inactivate both RB and p53. The best example occurs in adenovirus, which expresses E1A to inactivate RB, and E1B to inactivate p53. Expression of E1A alone causes apoptosis, which is suppressed by E1B[54]. These results are surprisingly similar to the suppression of apoptosis in *Rb*-null CNS neurons by the knockout of *Trp53* (REFS 7,8). Moreover, somatic inactivation of the mouse *Rb* gene in the cerebellar external granular layer cells of *Trp53*-null mice causes medulloblastoma[55]. These observations indicate that *Trp53* inactivation can provide conditions that allow subsequent *Rb* loss to promote tumour growth.

But what about human retinoblastoma? Hereditary and sporadic retinoblastoma results from the bi-allelic inactivation of *RB*, but does not acquire p53 mutation. There are two possible mechanisms by which retinoblastoma cells avoid apoptosis. In the first mechanism, RB only inhibits proliferation, but does not regulate apoptosis, in retinoblasts. In the second mechanism, in *RB*-null retinoblasts, apoptosis is prevented through epigenetic mechanisms, such as DNA methylation or gene amplification. Recent studies of retinoblastoma tumours have provided evidence for chromosomal aberrations outside the *RB* locus[56,57]. It will be of interest to determine if these additional aberrations provide survival signals for the

development of retinoblastoma. Unlike humans, the $Rb^{+/-}$ mice develop pituitary tumours with high penetrance[4–6,58,59]. By examining a large number of pituitary glands of $Rb^{+/-}$ mice, Lee and colleagues found that tissue apoptosis preceded tumour growth[58]. Retinoblastoma can develop in chimeric mice generated from $p107^{-/-}$, $Rb^{-/-}$ embryonic stem cells[60]. Increased apoptosis in the retina was also observed in these mice prior to tumour formation[60]. The anti-apoptosis mechanisms that drive the formation of *Rb*-null pituitary tumours and retinoblastoma are unknown at present.

In sporadic human cancers, the rare mutation of the *RB* gene, the increased phosphorylation of the RB protein and the combined presence of both RB and p53 mutations support the concept that RB is a conditional tumour suppressor. As RB can inhibit cell death, it should in theory also function as a conditional tumour promoter. This concept is supported by observations made in mice that express phosphorylation-resistant (constitutively active) variants of Rb in the mammary epithelial cells. One-third of these mice developed hyperplastic nodules, and ~7% of these mice developed adenocarcinomas[61]. In addition, we have recently observed that the *Rb-MI* allele, although having no effect on tumour incident by itself, can aggravate tumour development in *Trp53*-null mice (B.N.C. and J.Y.J.W., unpublished observations). The idea that RB can promote tumour development under specified circumstances might sound preposterous in light of our current understanding of RB function. However, given the important role of apoptosis in guarding against tumorigenesis, the inhibition of apoptosis by RB might be exploited in some cancer cells to promote malignant transformation.

Future directions

The ability of RB to inhibit apoptosis is now supported by many lines of evidence. Loss of *RB* through genetic mutation, viral oncoprotein sequestration or caspase-dependent degradation is, in each case, associated with the induction of apoptosis. Knockout of the *Rb* gene in mice sensitizes neurons and myocytes to apoptosis during development (FIG. 2). Through the creation of *Rb*-conditional knockouts, researchers have been able to dissociate Rb's effects on apoptosis and proliferation (TABLE 1). The transcription factor E2F, which interacts with RB, has been shown to regulate genes involved in apoptosis, including *Apaf1*, *Casp3*, *7*, *8* and *9* (REFS 12,19). Moreover, a degradation-resistant RB-MI can interfere with TNF-α-induced cytochrome *c* release and caspase activation (TABLE 1; BOX 1). So, RB is an inhibitor of cell death as well as an inhibitor of cell growth (FIG. 3b).

We propose two models to explain how cells choose to proliferate or to die following the inactivation of RB. Each model makes a previously untested prediction. The 'promoter-specific regulation' model predicts RB-assembled transcription complexes to remain on the promoters of apoptosis genes during S phase (FIG. 4). The 'context-dependent regulation' model predicts that caspases stimulate cell proliferation when sufficient survival signals are present (FIG. 4). Experiments to test predictions of these two models are feasible, and results would advance our understanding of how cells make the decision of life or death under complex physiological conditions.

The studies of RB have been focused on its regulation of transcription. However, RB does interact with a number of proteins that do not seem to participate in transcription regulation[37]. We found that the caspase-resistant Rb-MI can attenuate the transduction of death signal under conditions when transcription is irrelevant[29]. So, RB might inhibit apoptosis by mechanisms other than suppression of apoptosis gene expression. Whether RB inhibits apoptosis by post-transcriptional mechanisms remains to be determined.

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