Cell Death and Differentiation (1998) 5, 132–140
© 1998 Stockton Press All rights reserved 13509047/98 $12.00

Review

pRb and the Cdks in apoptosis and the cell cycle

Margaret M. Kasten[1,2] and Antonio Giordano[1]

[1] Departments of Pathology, Anatomy and Cell Biology, Sbarro Institute for Cancer Research and Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA
[2] corresponding author: Margaret M. Kasten

Received 16.6.97; revised 1.9.97; accepted 22.9.97
Edited by G. Melino

Abstract

Apoptosis is a fundamental biological process present in metazoan cells. Linking apoptosis to the cell cycle machinery provides a mechanism to maintain proper control of cell proliferation in a multicellular organism. pRb and the cyclin-dependent kinases may have dual roles as integral components of the cell cycle and regulators of apoptosis. In many instances manipulation of the cell cycle through these molecules can induce or inhibit apoptosis. Recent studies also identify pRb as a substrate for an apoptotic protease; however, other cell cycle components are not known substrates. While it is clear that many common molecules can affect cell proliferation and cell death, the universality of any one cell cycle molecule in apoptosis has yet to be determined.

Keywords: apoptosis; cell cycle; pRb; Cdc2; Cdks; cyclins

Abbreviations: cyclin dependent kinase (cdk); deoxyribonucleic acid (DNA); cytotoxic T lymphocytes (CTL); Chinese hamster ovary (CHO); retinoblastoma protein (pRb); tumor necrosis factor (TNF); poly(ADP-ribose) polymerase (PARP); kilodalton (kDa)

Introduction

Balance between cell proliferation and cell death is imperative for homeostasis in multicellular organisms. Somatic cells proliferate and divide by executing the cell cycle. Cells can die by necrotic mechanisms, but programmed cell death, or apoptosis, allows an organism to eliminate unwanted cells through a safe, orderly process. Apoptosis is crucial for the elimination of unwanted cells during normal development of embryo limbs, neurogenesis, and the elimination of self-reactive T lymphocytes (see Jacobson *et al*, 1997 for review). Furthermore, coupling the cellular processes of proliferation and apoptosis might provide a means by which an organism can regulate cell expansion.

Several lines of reasoning have fostered the idea of a link between apoptosis and the cell cycle. First, some of the crude morphological changes occurring during apoptosis are reminiscent of mitosis (see King and Cidlowski, 1995 for review). Mitosis and apoptosis are both characterized by a loss of substrate attachment, condensation of chromatin, and phosphorylation and disassembly of nuclear lamins. Apoptotic cells however, continue on the path of self-destruction with the rapid onset of nuclear fragmentation and membrane blebbing, characteristics mitotic cells don't share. A second and more compelling argument for a link between the cell cycle and apoptosis comes from the growing evidence indicating that the manipulation of the cell cycle may either prevent or induce an apoptotic response (see Evan *et al*, 1995 for review). Much of this data focuses on the role of molecules in late G1, in particular the p53 and pRb proteins. However, there is increasing interest in other cell cycle components in apoptosis.

Since the apoptosis and the cell cycle fields are each vast and complex, we will refer the reader to various review articles for additional discussion. This review will focus on the role of pRb and the various cyclin-dependent kinases (Cdks) in apoptosis. The involvement of p53 in apoptosis and cell cycle is well established (Enoch and Norbury, 1995; Levine, 1997), and we will discuss its role in reference to the previously mentioned cell cycle components.

Cell cycle and the Cdks

Cell proliferation is controlled by a network of interconnected extracellular and intracellular signaling pathways (see MacLachlan *et al*, 1995 for review). These signals ultimately impinge on the cyclin dependent kinases (Cdks), a family of kinases that drive cells from one phase of the cell cycle to the next. As indicated by their name, cyclin dependent kinases rely upon a cyclin partner for enzymatic activity. These cyclin-Cdk complexes phosphorylate substrates required for progression through the cell cycle. Once bound by their cyclin partner, subsequent activity is regulated by both activating and inhibitory phosphorylation on the Cdk subunit. Association of the cyclin-Cdk complexes with various inhibitory molecules achieves further regulation of their kinase activity.

The cell cycle consists of a round of chromosomal DNA replication in S phase followed by segregation of the chromosomes into two daughter cells during M phase. The period, or gap, separating the M and S phases is known as G1, while the period between S and M is referred to as G2. Cells undergoing differentiation exit the cell cycle during G1 to enter into G0, a quiescent state. The Cdc2 kinase, the first characterized Cdk, forms complexes with cyclins A and B, which are crucial for the cell to progress into M phase.

Subsequent passage through G1 into S phase is controlled by Cdks that are sequentially regulated by cyclins D, E and A. One of the primary targets of these G1 kinases is the pRb family of proteins, which will be discussed later. The kinase partners of the D-type cyclins are Cdk4 and Cdk6. The cyclin D-Cdk complexes are crucial to the cell cycle because they couple extracellular signals to the cell cycle (Peeper *et al*, 1997). However, they are no longer required once cells pass the late G1 restriction, or R, point (Sherr, 1995). Once a cell passes the R point, it becomes committed to entering S phase and to that round of the cell cycle. The catalytic partner to cyclin E is the Cdk2 kinase, which is also activated by cyclin A later in G1. The cyclin E and A complexes in late G1 are important to move the cell into S phase. Checkpoints are superimposed on the basic cyclin-Cdk cycle to ensure that certain processes in the cycle are completed before beginning the next process (see Elledge, 1996 for review).

Cdks in apoptosis

Several lines of evidence have suggested an involvement of the mitosis-promoting kinase, Cdc2, in the process of apoptosis. Early observation showed that eukaryotic cells overexpressing Cdc2 at an inappropriate time during the cell cycle results in mitotic catastrophe (Heald *et al*, 1993; Lundgren *et al*, 1991; Russel and Nurse, 1987). Mitotic catastrophe occurs when cell cycle components are mutated or overexpressed, leading to premature entry into mitosis and death. Mitotic catastrophe morphologically resembles apoptosis in several respects (see King and Cidlowski, 1995 for review) and this prompted investigations into the role of Cdc2 and other Cdks in apoptosis.

Perhaps the most convincing argument for a Cdc2 role in apoptosis comes from a model of apoptosis in cytotoxic T lymphocytes (CTL) (Shi *et al*, 1994). CTL cells activate apoptosis through granzyme B, a serine protease, and apoptosis induced by granzyme B has been recently reviewed (Greenberg, 1996). YAC-1 lymphoma cells underwent apoptosis after treatment with granzyme B and perforin, a pore forming compound. Apoptosis induced in this system was accompanied by a dramatic increase in Cdc2 kinase activity. In addition, Cdc2 activity blocked by pseudosubstrate peptides also blocked apoptosis (Shi *et al*, 1994). This strongly indicates that Cdc2 kinase activity is required for the observed apoptosis. Further support was provided by using FT210, a cell line expressing a temperature-sensitive Cdc2 protein that prematurely degrades at 39°C (Th'ng *et al*, 1990). FT210 cells resisted apoptosis when exposed to granzyme B and perforin after pre-incubation at 39°C but not at the permissive temperature (Shi *et al*, 1994). Therefore, granzyme B-induced apoptosis requires Cdc2.

The requirement for Cdc2 activity is not restricted to granzyme B pathways. Staurosporine, a protein kinase inhibitor, induces an apoptotic pathway negatively regulated via bcl-2 (Jacobson *et al*, 1993). FT210 cells treated with staurosporine induce apoptosis in a Cdc2-dependent manner (Shi *et al*, 1994). Cyclin A-Cdc2 activation is also observed in HIV-1 Tat-induced apoptosis (Li *et al*, 1995), while cyclin B1-Cdc2 kinase activity is increased in human promyelocytic leukemia cells, HL60, prior to apoptosis (Shimizu *et al*, 1995). Likewise, T-cell death by anti-CD3 antibodies requires Cdc2 kinase activity (Fotedar *et al*, 1995). Together these observations support the model that Cdc2 is crucial for cell death in these systems.

However, Cdc2 cannot be a universal regulator of apoptosis. While FT210 cells require Cdc2 for apoptosis by granzyme B or staurosporine, FT210 cells exposed to a variety of other apoptosis-inducing agents do not require Cdc2 for apoptosis (Martin *et al*, 1995). A very broad range of agents was examined including inhibitors of RNA synthesis, protein synthesis, topoisomerase II, as well as hydrogen peroxide and ultraviolet light (Martin *et al*, 1995). These results indicate that Cdc2 cannot be an essential component for all apoptosis. Indeed, Cdc2 is not activated in apoptosis of serum starved fibroblast (Oberhammer *et al*, 1994) or in postmitotic neurons (Freeman *et al*, 1994). Cdc2 enzymatic activity is also not critical for Fas-induced cell death of the human T lymphoma cell line, HUT-78 (De Luca *et al*, 1997). There are even instances where Cdc2 activation actually results in protection from apoptosis (Ongkeko *et al*, 1995).

While it is clear that Cdc2 is not an essential component for all apoptosis, this does not rule out the involvement of other kinases. Cyclin A-Cdk2, as well as cyclin A-Cdc2, is induced after treatment with HIV-1 Tat, and apoptosis can be inhibited with antisense oligonucleotides to cyclin A (Li *et al*, 1995). This indicates that one or both of these two cyclin A-Cdk complexes are required to mediate apoptosis in this system. Indeed, it has been argued that activation of cyclin A-Cdk complexes may be a required step for apoptosis (Meikrantz *et al*, 1994).

Cyclin D complexes may also play a part of some apoptotic pathways. A specific induction of cyclin D1 expression was found in neurons undergoing apoptosis (Freeman *et al*, 1994; Kranenburg *et al*, 1996), indicating that cyclin D1, and perhaps the other D-type cyclins, have dual roles in proliferation and in cell death. Sofer-Levi and Resnitzky found that expressing cyclin D1 from an inducible promoter in serum starved rat fibroblasts leads to apoptotic cell death, while expression of cyclin E is not sufficient to cause apoptosis (Sofer-Levi and Resnitzky, 1996). Likewise, increased expression of cyclin D1 in mammary epithelial cells enhanced apoptosis when cells were exposed to serum starvation or hydroxyurea treatment (Han *et al*, 1996). Others have found that cyclin D3 sensitizes tumor cells to TNF-induced apoptosis (Janicke *et al*, 1996), further supporting the idea that other cyclin complexes may be involved in mediating an apoptotic response.

Other data strongly argues for the involvement of the Cdk-related kinase PITSLRE, in apoptosis. Ectopic expression of PITSLRE in Chinese hamster ovary (CHO) cells is sufficient to induce apoptosis (Lahti *et al*, 1995). It is also very interesting that cell death in Fas-activated T cells is associated with elevated PITSLRE kinase activity. This activity is blocked by serine protease inhibitors that suppress apoptosis (Lahti *et al*, 1995). These results suggest that the PITSLRE kinase may lie within apoptotic

signaling pathways and that the kinase itself may be activated by one of the apoptotic proteases. Indeed, recent evidence indicates that PITSLRE is cleaved during TNF-mediated apoptosis in cell culture and that two different apoptotic proteases can produce the observed cleavage *in vitro* (Beyaert *et al*, 1997). While PITSLRE may play a role in apoptosis, it should be stated that the PITSLRE kinase has no known cyclin partner and its involvement in the cell cycle is uncertain.

The fact that different apoptotic inducers require no single Cdk complex suggests these kinases are not part of a common apoptotic pathway. One hypothesis is that the Cdks affect the sensing mechanisms for some apoptotic inducers, but not others (Figure 1). These different pathways then converge on a common apoptotic pathway. In this model the Cdks affect the decision to begin apoptosis, but not the apoptotic program itself.

The role of pRb and E2F in the cell cycle

The retinoblastoma protein, pRb, acts to connect the cell cycle to the transcriptional machinery primarily by binding to the E2F transcription factors, although associations with other proteins are important for pRb function (Taya, 1997; Weinberg, 1995). E2F activity is now known to depend on heterodimers composed of an E2F protein bound to a DP family member (Lam and La Thangue, 1994; Muller, 1995). For simplicity we will refer to these complexes collectively as E2F.

pRb permits the cell cycle machinery to regulate expression of a myriad of genes that are required to advance the cell from G1 to the S phase. The ability of pRb to bind and regulate proteins is largely determined by its phosphorylation state. When pRb is hypophosphorylated, it is capable of associating with its binding partners, including E2F. This binding prevents E2F from activating its target genes (Helin *et al*, 1993). In fact, pRb bound to E2F actively represses transcription (Lam and Watson, 1993; Weintraub *et al*, 1992, 1995). Phosphorylation of pRb results in the release of E2F and the subsequent activation of genes necessary for DNA synthesis. Several of the G1 cyclin-Cdk complexes target the pRb protein for phosphorylation (see Taya, 1997 for review) (Figure 2A). Indeed, a variety of cell cycle components and regulators directly or indirectly affect pRb activity (Table 1). Without this phosphorylation pRb holds the cell in G1, preventing further advancement through the cell cycle.

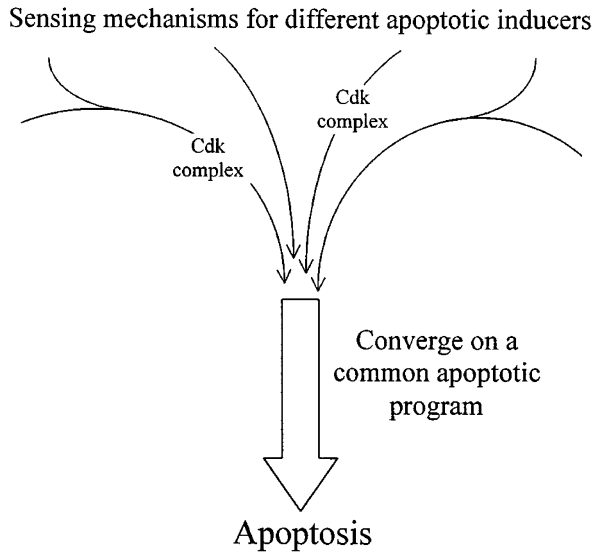


Figure 1 A model depicting Cdk involvement in apoptosis

pRb and E2F in apoptosis

Studies of small DNA tumor viruses suggest that inducers of cell proliferation could mediate apoptosis. In adenovirus E1A is the principal early gene required to drive the host cell proliferation necessary for viral replication. E1A binds to pRb preventing it from associating with E2F (Bagchi *et al*, 1992). This in turn leads to activation of DNA synthesis and cell proliferation. However, in the absence of E1B, the second early viral gene, E1A induces apoptosis (White *et al*, 1991) (Figure 2C). The same E1A domains required for induction of apoptosis coincide with those domains necessary for cell proliferation (Debbas and White, 1993), and these same regions are needed for association with the pRb protein. Similar studies have examined the E7 protein of human papilloma virus and the large T antigen of SV40; both proteins bind to pRb and have been shown to induce apoptosis *in vivo* (Howes *et al*, 1994; Naik *et al*, 1996), further suggesting an overlap between cell cycle and apoptotic functions.

Several lines of evidence support the hypothesis that pRb itself acts as a negative regulator of apoptosis. Homozygous pRb null mice die *in utero* after 12–13 days of development and exhibit massive apoptosis in tissues known to normally express high levels of pRb (Clarke *et al*, 1992; Jacks *et al*, 1992; Lee *et al*, 1992). In particular, inappropriate apoptosis has been documented in the developing lens of pRb deficient mice (Howes *et al*, 1994; Morgenbesser *et al*, 1994).

Experiments using the SAOS-2 cell line also support pRb as an inhibitor of apoptosis. Ionizing radiation induces apoptosis in SAOS-2 cells, a cell line lacking pRb expression. SAOS-2 cells expressing exogenous wild-type pRb become resistant to apoptosis when exposed to radiation (Haas-Kogan *et al*, 1995). Furthermore, mutant pRb proteins that do not bind the E1A or E2F proteins do not protect cells from apoptosis (Haas-Kogan *et al*, 1995). These data support the hypothesis that E1A induces apoptosis by interfering with the protective functions of pRb, which include inactivating the E2F transcription factor.

E1A's inhibition of pRb activity ultimately affects E2F activity, leading to the suggestion that E2F may initiate apoptosis. This has been further demonstrated in two sets of experiments. First, three separate groups have shown that when constitutively expressed in mouse embryo fibroblast cell lines, the E2F-1 transcription factor was capable of inducing apoptosis after passage through S phase (Qin *et al*, 1994; Shan and Lee, 1994; Wu and Levine, 1994). In addition, mouse 'knockout' experiments

show that E2F-1 homozygous null mice demonstrate increased cell proliferation and neoplasias in several tissues as the mice age (Field *et al*, 1996; Yamasaki *et al*, 1996).

The results from the E2F-1 null mice were quite unexpected, as E2F has generally been proposed to promote cell proliferation, not reduce it. However, most of the studies examining E2F function have relied on overexpression of either wild-type or mutant E2F and protein concentration could prove crucial to cellular function. This may be especially so in the case of E2F as it is bound by pRb. When overexpressed, E2F will likely be deregulated during early G1, as there is more protein than can be bound by its normal pRb regulator. As predicted by this hypothesis, apoptosis induced by E2F-1 overexpression in fibroblasts can be suppressed by coexpression of wild-type pRb (Qin *et al*, 1994). This supports the scenario that pRb exerts its effects on apoptosis by regulating E2F activity; however, it does not eliminate the possibility that pRb suppresses apoptosis through additional mechanisms.

pRb and p53

The p53 gene is a common target for genetic alterations in human cancer (Friend, 1994; Levine, 1997). The p53 protein has the important role of ensuring that when exposed to DNA damaging agents, cells undergo cell cycle arrest (Figure 1B) and attempt to repair the damage, typically, but not exclusively, in G1. In response to DNA damage the p53 protein is activated and turns on transcription of the p21 gene. p21 regulates the activity of most cyclin-Cdk complexes, including those that phosphorylate pRb. Interestingly, it is the number of p21 molecules associated with a complex that determines kinase activity (Zhang *et al*, 1994). The end result of this pathway is that pRb remains hypophosphorylated, in which state it inactivates E2F.

Mice homozygous null for p53 exhibit a predisposition to cancer (Donehower *et al*, 1992, 1995). While the observation of multiple malignancies in p53 mutant mice could be due to the proliferation of cells with DNA damage, the ability of wild-type p53 to induce apoptosis when reintroduced into p53-deficient cells demonstrates a role for p53 in reducing neoplasias through apoptosis (Symonds *et al*, 1994). There is also increasing evidence that p53 is required for much, but not all, of the apoptosis seen in pRb-deficient or E2F-1 overexpressing cells (Howes *et al*, 1994; Morgenbesser *et al*, 1994; Qin *et al*, 1994; Wu and Levine, 1994). Similarly, overexpression of wild-type p53 in HeLa cells, which have very low levels of p53 and pRb, is sufficient to induce apoptosis (Haupt *et al*, 1995). An excess of the pRb protein, in this instance, overcomes apoptosis, (Haupt *et al*, 1995). However, pathways separate from p53 must also be investigated when considering pRb and cell death since pRb-deficient and E2F-induced apoptosis is not strictly p53 dependent. These pathways have yet to be elucidated.

Taken together, these results suggest that p53 and pRb may have opposing roles in the control of apoptosis. Wild-type pRb may actually serve as a barrier to p53-mediated apoptosis (Yonish-Rouach *et al*, 1993). This suggests a reason why the simultaneous inactivation of both p53 and pRb is frequently required for viral transformation and replication. In adenovirus, the E1A protein binds and inactivates pRb, while E1B inactivates p53 (Moran, 1993). The human papilloma virus has the E6 and E7 proteins which target p53 and pRb, respectively (Vousden, 1993). The finding that overexpressing viral E1A or E7 alone induces apoptosis (Howes *et al*, 1994; White *et al*, 1991) (Figure 2C), shows that viruses have a dual problem. Not only must they inappropriately trigger cells to re-enter the cell cycle by inactivating pRb, but they must also keep the cell alive long enough to complete the viral life cycle. To prevent the cell from undergoing apoptosis, adenovirus inactivates p53 through E1B (Debbas and White, 1993) and human papilloma virus inactivates p53 through E6 (Scheffner *et al*, 1990) (Figure 2D). A similar situation exists for tumor cells. p53 and pRb are commonly found to be inactive in tumor cells, thereby allowing these cells to proliferate uncontrollably without undergoing apoptosis (Williams *et al*, 1994).

Table 1 Cell cycle effectors in apoptosis and their relationship to pRb

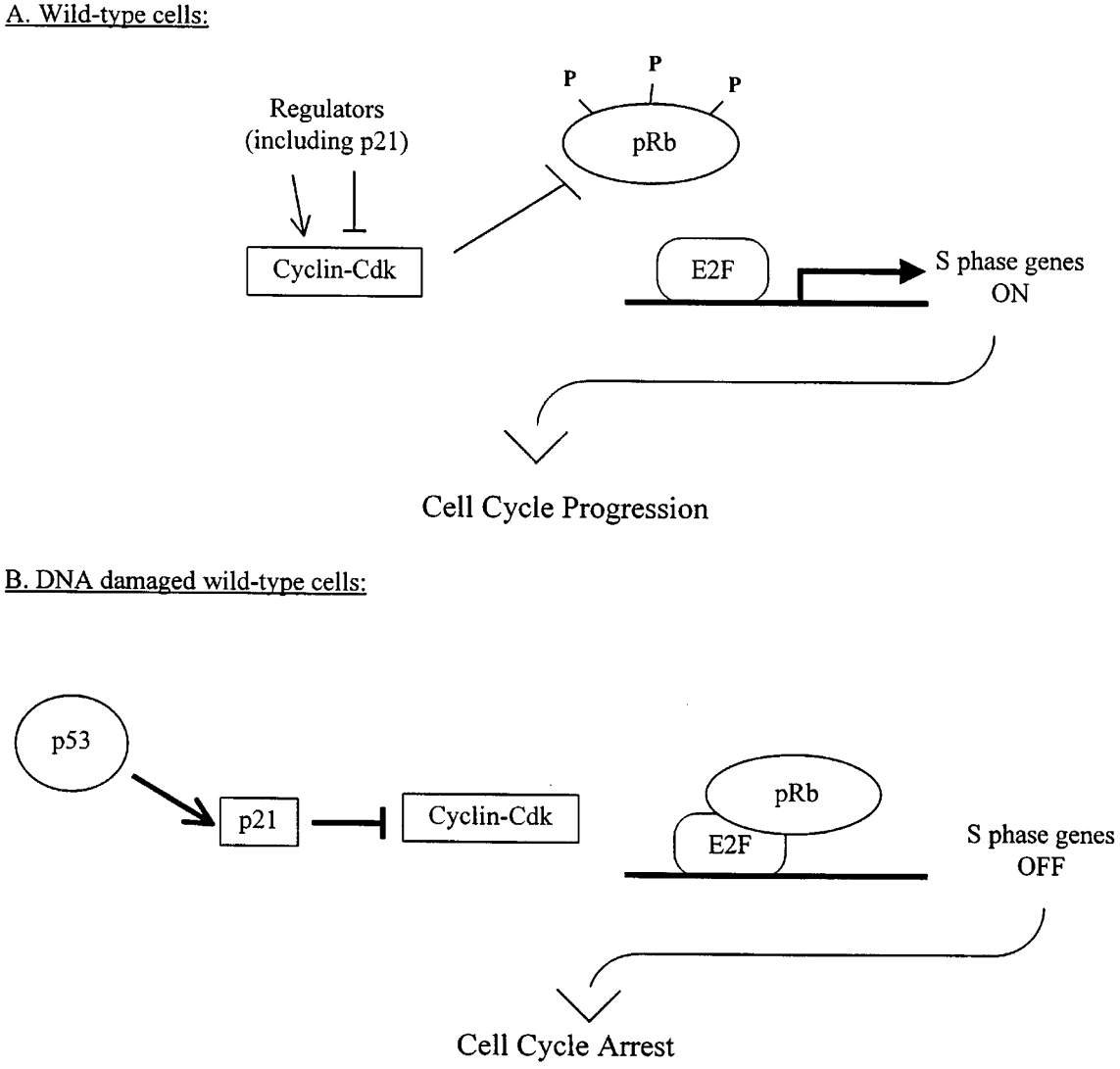
	Gene	Cell cycle role	Effect on or by pRb
Inhibit Apoptosis:			
	pRb	G1 checkpoint Inhibits E2F	
Promote Apoptosis:			
	p53	G1 and G2 checkpoint Induces p21	Indirectly activates pRb
	p21	Inhibits cell cycle kinases	Indirectly activates pRb
	E2F	Transcription factor Activates S phase genes	Inactivated by pRb
	Cdc2	G2 kinase Promotes M phase	None known
	Cdk2	G1 kinase Promotes S phase	Inhibitory phosphorylation of pRb
	Cyclin A	Activates G1 and G2 kinases	Inhibitory phosphorylation of pRb
	Cyclin D	Activates G1 kinases Responds to mitogens	Inhibitory phosphorylation of pRb
	E1A	Promotes S phase	Inactivates Rb
	c-myc	Transcription factor Promotes S phase	None known

The mechanism by which pRb suppresses apoptosis is not clear. One hypothesis on how pRb prevents apoptosis is by preventing S phase entry, thereby promoting a quiescent state. In this quiescent state cells would not undergo unscheduled DNA synthesis and, therefore, would avoid accumulation of DNA damage that might trigger apoptosis. If so, then one might expect aphidicolin to protect cells. Aphidicolin is an inhibitor of DNA polymerase α that arrests cells at the G1/S border. However, simply arresting cell cycle progression by inhibiting DNA polymerase is not sufficient to suppress apoptosis (Haas-Kogan *et al*, 1995). The mechanism by which pRb rescues cells from cell death must be more complex.

The decision to enter into apoptosis or cell cycle arrest is affected by many factors. The presence of pRb and its antiproliferative function inhibits growth in ways normally associated with DNA damage. In the absence of this effect, damaged cells apoptose after receiving inappropriate signals to proceed through the cell cycle. This coupling of proliferative signals to apoptosis may provide the organism with one final opportunity to rid itself of potentially dangerous cells. As long as this coupling is intact, genetic alterations allowing inappropriate cell proliferation will also increase cells following apoptotic pathways. Loss of this coupling by mutations in critical cell cycle genes might reduce cell death and could in turn lead to tumor progression.

pRb as a target of caspase protease(s)

Members of the interleukin 1β-converting enzyme family of proteases, also known as caspases, are important mediators of programmed cell death (see Kumar and Lavin, 1996 for review). Cleavage of specific proteins by caspases is associated with the many different morphological changes that occur during apoptosis, and it may be that cleavage of at least some of these substrates is responsible for some of these changes. Several nuclear substrates for these enzymes have been identified. They include poly(ADP-ribose) poly-



merase (PARP), lamins, topoisomerases, and DNA-dependent protein kinase (DNA-PK) (Kumar and Lavin, 1996). While PARP plays an important role in genome maintenance (Lindahl *et al*, 1995), it is unlikely that cleavage of PARP is a principal event in apoptosis since PARP-deficient mice show normal resistance to DNA-damaging agents. This suggests that the apoptotic process is intact (Wang *et al*, 1995). However, the number of substrates that must be cleaved for the cell to undergo apoptosis is not known since cleavage of a single substrate has not been demonstrated to cause cell death. This may be due to apoptosis requiring the cleavage of multiple substrates or that other substrates still need to be identified.

In the past year several groups have identified pRb as a potential substrate for caspase-like proteases (An and Dou, 1996; Chen *et al*, 1997; Dou *et al*, 1997; Janicke *et al*, 1996). It has been reported that pRb was cleaved to 68 kDa and 48 kDa fragments in HL-60 and U937 cells undergoing drug-induced apoptosis (An and Dou, 1996). Fas-induced apoptosis induced similar pRb proteolysis in

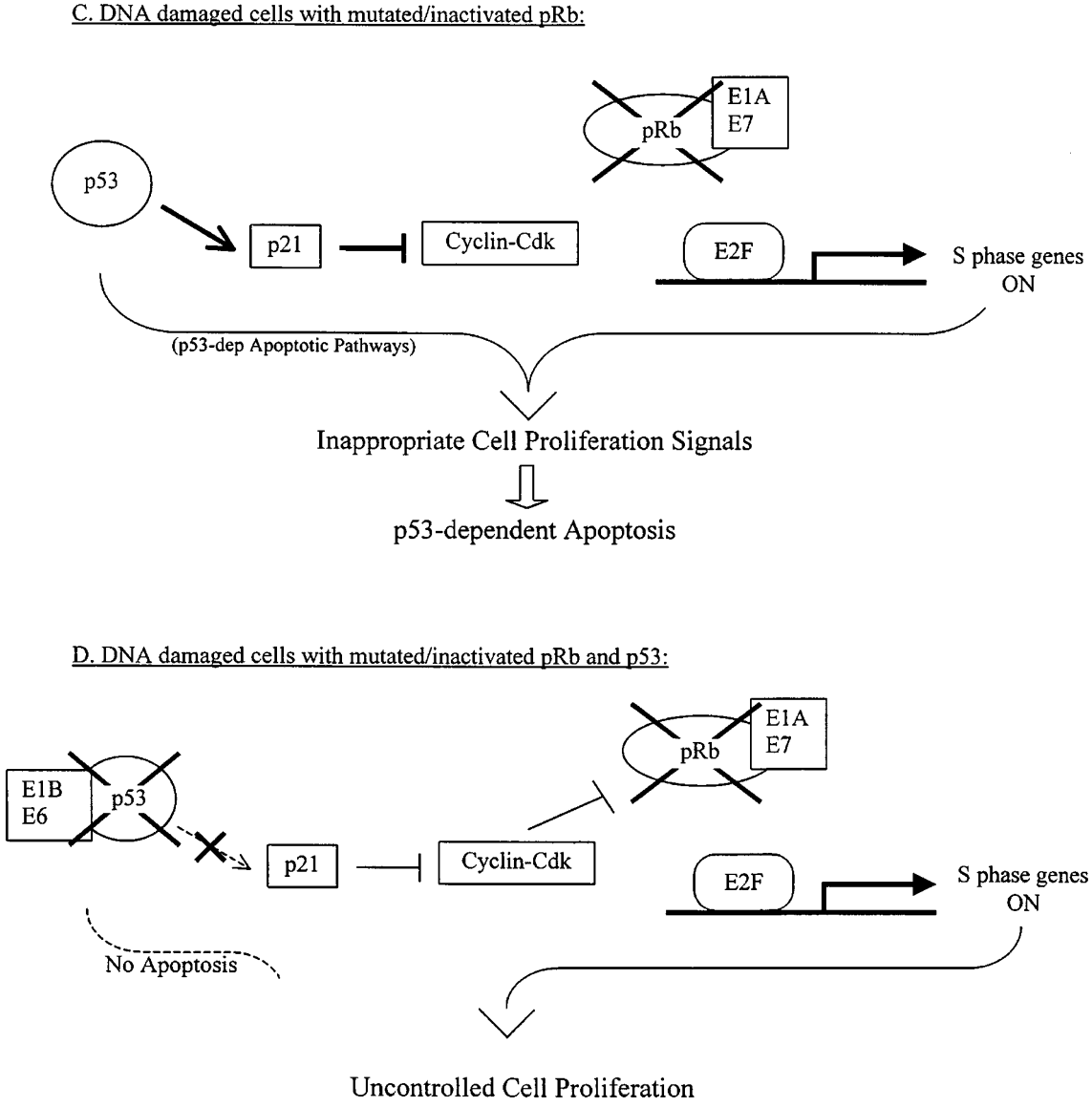


Figure 2 The relationship between p53, pRb and E2F in the cell cycle and apoptosis. (**A**) In a wild-type cell the activity of cyclin-Cdk complexes is modulated by both positive and negative regulators, thereby allowing cyclin-Cdk complexes to phosphorylate pRb during G1. Hyperphosphorylated pRb does not bind to the E2F transcription factor resulting in the transcription of genes required for S phase and cell cycle progression. (**B**) Cells with DNA damage have stabilized p53, which induces the p21 promoter. The increased levels of p21 inhibit cyclin-Cdk activity and leads to hypophosphorylated pRb. This form of pRb binds to E2F repressing transcription of S phase genes, and results in cell cycle arrest. (**C**) DNA damaged cells with inactive pRb (through mutation or viral oncoproteins) inappropriately activate S phase genes. These signals to inappropriately replicate DNA are sensed by p53 and lead to apoptosis. (**D**) DNA damaged cells with inactivated pRb and p53 (through mutation or viral oncoproteins) inappropriately activate S phase genes, however, they do not have p53 to induce apoptosis. This results in uncontrolled cell growth

Jurkat cells (Dou *et al*, 1997). It has been suggested that a caspase protease controls this pRb cleavage since a specific tetrapeptide caspase inhibitor prevents cleavage (An and Dou, 1996; Dou *et al*, 1997). The significance of this particular pRb cleavage is unclear. Indeed, this cleavage may not be restricted to cells undergoing apoptosis. A 68 kDa pRb species is reported in various tumor cell lines independent of apoptosis (Chen *et al*, 1997). The 68 kDa peptide in these tumors is able to bind E2F-1, and so retains some of its normal functions. It should be noted, however, that the 68 kDa fragments identified by each group may not be identical, although both correspond to the N-terminal region of pRb.

The pRb protein undergoes a much different cleavage during other forms of apoptosis. Apoptosis is associated with cleavage of 42 amino acids from the C-terminus of pRb (Chen *et al*, 1997; Janicke *et al*, 1996). Cleavage is blocked *in vivo* and *in vitro* by two specific inhibitors of caspase-like proteases (Janicke *et al*, 1996). An *in vitro* point mutation within the predicted caspase-like cleavage site also inhibited pRb proteolysis (Janicke *et al*, 1996), further supporting the hypothesis that a caspase-like protease is responsible for pRb cleavage. Cleaving the C-terminal 42 amino acids causes pRb to migrate at a rate that mimics hypophosphorylated pRb (Chen *et al*, 1997). Therefore, claims that dephosphorylation of pRb occurs during apoptosis (An and Dou, 1996; Dou *et al*, 1997) must be reexamined, as dephosphorylation was inferred by protein migration on polyacrylamide gels.

What is the functional consequence of removing 5 kDa from the C-terminus of pRb? This truncated pRb species retains its ability to bind many of its known protein partners. The cleaved pRb binds D-type cyclins (Janicke *et al*, 1996) as well as E2F-1 (Chen *et al*, 1997; Janicke *et al*, 1996). However, this truncated form of pRb failed to bind the Mdm2 protein (Janicke *et al*, 1996). Mdm2 is known to bind to and downregulate the G1 arrest activity of p53 (Levine, 1997), but Mdm2 also binds and inhibits pRb activity (Xiao *et al*, 1995). The consequence of the loss of Mdm2 binding by pRb has not been determined. Further experiments will be required to determine whether other pRb functions are altered with this cleavage.

The identification of pRb as a substrate for a caspase provides an additional means by which cells may link the cell cycle to apoptosis. Further experiments are required to determine whether C-terminal pRb cleavage is common to all cells undergoing apoptosis and the biological consequences of this cleavage. Determining the identity of the caspase responsible for cleavage would lend added weight to these findings. The use of purified caspase proteases in an *in vitro* system should readily address this question.

Concluding remarks

Cell proliferation and apoptosis are intrinsically linked, if not in all situations, at least in many. There are a variety of molecules other than pRb, p53, and the Cdks that are candidates for roles in both cell cycle and apoptosis. For instance, the INK4 family of genes inhibits cyclin D-associated kinase activity and, therefore, effects the phosphorylation state of pRb. Since pRb and cyclin D have connections to apoptosis, one would predict that INK4 affects cell death as well. Indeed, mutations in one of these three genes are frequently found in human cancers and it has been proposed that inactivation of this pathway may be essential for tumor development (Sherr, 1996). Other molecules, such as c-*myc*, are also clearly involved in both cell proliferation and apoptosis (see Evan *et al*, 1995 for review). Constitutively expressing c-*myc* in serum starved Rat-1 fibroblasts stimulates both cell proliferation and apoptosis (Evan *et al*, 1992). However recent evidence indicates that these are two independent events. It appears that the c-*myc* induced apoptosis and S phase progression are mediated by two distinct molecular pathways that occur in response to induction of c-*myc* (Rudolph *et al*, 1996).

Other candidate molecules include pRb's two relatives, p107 and pRb2/p130 (see Paggi *et al*, 1996 for a review of the Rb family). The E1A protein targets all three members of the pRb family for inactivation, and so experiments examining apoptosis by E1A (and other small DNA tumor virus proteins) necessarily involve all three family members. Apart from these experiments only pRb has been examined in apoptosis. However, mouse models demonstrate functional overlap in the apoptotic process between pRb and p107 in the liver and central nervous system (Lee *et al*, 1996). Recent evidence indicates that even though the retinoblastoma family members are able to complement each other, they are not fully functionally redundant (Claudio *et al*, 1994, 1996). Further work is necessary to examine the areas of convergence and divergence between p107 and pRb2/p130. This information holds the potential for developing better cancer therapies designed for specific organs or tissues.

An additional point that should be made is that the cell cycle, and therefore proliferation, is regulated in part by checkpoints. Cell cycle checkpoints are commonly referred to as regulatory pathways that control the order and timing of cell cycle transitions (see Elledge, 1996; Nasmyth, 1996 for reviews). These pathways act as surveillance mechanisms to arrest the cell cycle in response to damage, thereby providing sufficient time for repair before progressing to the next stage of the cycle. DNA damage is a well studied inducer of both cell cycle arrest and apoptosis. The p53 protein responds to DNA damage by either enforcing cell cycle arrest or triggering apoptosis and is a cell cycle checkpoint molecule. Checkpoints in general could be an additional connection between the cell cycle and apoptosis. Checkpoints are important to chemotherapies designed to eliminate cancer cells. Many agents appear to kill cancer cells by activating checkpoint-mediated apoptosis (Hartwell and Kastan, 1994). Intriguingly, p53 is a substrate for Cdc2 complexes (Milner *et al*, 1990; Sturzbecher *et al*, 1990), providing an additional connection between the actual cell cycle machinery and this checkpoint protein. It would be interesting to know whether Cdk complexes regulate other mammalian checkpoint molecules.

It is reasonable to believe that cells connect cell proliferation and cell death through common molecules. However, the precise mechanism of the link is yet unclear. Are components of the cell cycle machinery also

components of the apoptotic machinery or are cell cycle molecules acting as sensors or triggers to induce a lethal pathway when necessary? What is the role of cell cycle checkpoints in apoptosis? In any case, linking the cell cycle to apoptosis would facilitate the removal of cells defective in cell cycle regulation. This, as seen in cancer, is crucial to an organism's survival.

Acknowledgements

We wish to thank the members of the Giordano lab for stimulating discussions pertaining to this topic. We especially thank Drs Robert Mecklenburg and Peter Stiegler for critically reading this manuscript and their insightful comments.

References

An B and Dou QP (1996) Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 beta-converting enzyme-like protease as candidate. Cancer Res. 56: 438 – 442

Bagchi S, Weinmann R and Raychaudhuri P (1992) The retinoblastoma protein copurifies with E2F-1, an E1A-regulated inhibitor of the transcription factor E2F. Cell 65: 1053 – 1061

Beyaert R, Kidd VJ, Cornelis S, van de Craen M, Denecker G, Lahti JM, Gururajan R, Vandenabeele P and Fiers W (1997) Cleavage of PITSLRE kinases by ICE/CASP1 and CPP32/CASP-3 during apoptosis induced by tumor necrosis factor. J. Biol. Chem. 272: 11694 – 11697

Chen W-D, Otterson GA, Lipkowitz S, Khleif SN, Coxon AB and Kaye FJ (1997) Apoptosis is associated with cleavage of a 5 kDa fragment from RB which mimics dephosphorylation and modulates E2F binding. Oncogene 14: 1243 – 1248

Clarke A, Maandag E, Van Roon M, Van der Lugt N, Van der Valk M, Hooper M, Berns A and Te Riele H (1992) Requirement for a functional Rb-1 gene in murine development. Nature 359: 328 – 330

Claudio PP, De Luca A, Howard CM, Baldi A, Firpo EJ, Koff A, Paggi MG and Giordano A (1996) Functional analysis of pRb2/p130 interaction with cyclins. Cancer Res. 56: 2003 – 2008

Claudio PP, Howard CM, Baldi A, De Luca A, Fu Y, Condorelli G, Sun Y, Colburn N, Calabretta B and Giordano A (1994) p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. Cancer Res. 54: 5556 – 5560

De Luca A, De Maria R, Baldi A, Trotta R, Facchiano F, Giordano A, Testi R and Condorelli G (1997) Fas-induced changes in cdc2 and cdk2 kinase activity are not sufficient for triggering apoptosis in HUT-78 cells. J. Cell. Biochem. 64: 579 – 585

Debbas M and White E (1993) Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. 7: 546 – 554

Donehower LA, Godley LA, Aldaz M, Pyle R, Shi Y-P, Pinkel D, Gray J, Bradley A, Demina D and Varmus HE (1995) Deficiency of p53 acclerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev. 9: 882 – 895

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CAJ, Butel JS and Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356: 215 – 221

Dou QP, An B, Antoku K and Johnson DE (1997) Fas stimulation induces RB dephosphorylation and proteolysis that is blocked by inhibitors of the ICE protease family. J. Cell. Biochem. 64: 586 – 594

Elledge SJ (1996) Cell cycle checkpoints: preventing and identity crisis. Science 274: 1664 – 1671

Enoch T and Norbury C (1995) Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis, and the roles of p53 and ATM. Trends Biochem. Sci. 20: 426 – 430

Evan GI, Brown L, Whyte M and Harrington E (1995) Apoptosis and the cell cycle. Curr. Opin. Cell Biol. 7: 825 – 834

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC (1992) Induction of apoptosis in fibroblasts by c-myc protein. Cell 69: 119 – 128

Field SJ, Tsai F-Y, Kuo F, Zubiaga AM, Kaelin WG-J, Livingston DM, Orkin SH and Greenberg ME (1996) E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 85: 549 – 561

Fotedar R, Flatt J, Gupta S, Margolis RL, Fitzgerald P, Messier H and Fotedar A (1995) Activation-induced T-cell death is cell cycle dependent and regulated by cyclin B. Mol. Cell. Biol. 15: 932 – 942

Freeman RS, Estus S and Johnson EM (1994) Analysis of cell cycle-regulated gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. Neuron 12: 343 – 355

Friend S (1994) p53: a glimpse at the puppet behind the shadow play. Science 265: 334 – 335

Greenberg AH (1996) Activation of apoptosis pathways by granzyme B. Cell Death Differ 3: 269 – 274

Haas-Kogan DA, Kogan SC, Levi D, Dazin P, A TA, Fung YK and Israel MA (1995) Inhibition of apoptosis by the retinoblastoma gene product. EMBO J. 14: 461 – 472

Han EK, Begemann M, Sgambato A, Soh JW, Doki Y, Xing WQ, Liu W and Weinstein IB (1996) Increased expression of cyclin D1 in a murine mammary epithelial cell line induces p27kip1, inhibits growth, and enhances apoptosis. Cell Growth Differ. 7: 699 – 710

Hartwell LH and Kastan MB (1994) Cell cycle control and cancer. Science 266: 1821 – 1828

Haupt Y, Rowan S and Oren M (1995) p53-mediated apoptosis in HeLa cells can be overcome by excess pRB. Oncogene 10: 1563 – 1571

Heald R, McLoughlin M and McKeon F (1993) Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase. Cell 74: 463 – 474

Helin K, Harlow E and Fattaey A (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. 13: 6501 – 6508

Howes KA, Ransom N, Papermaster DS, Lasudry JG, Albert DM and Windle JJ (1994) Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. Genes Dev. 8: 1300 – 1310

Jacks T, Fazeli A, Schmitt E, Bronson R, Goodell M and Weinberg R (1992) Effects of an Rb mutation in the mouse. Nature 359: 295 – 300

Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC and Raff MC (1993) Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature 361: 365 – 369

Jacobson MD, Weil M and Raff MC (1997) Programmed cell death in animal development. Cell 88: 347 – 354

Janicke RU, Lin XY, H LF and Porter AG (1996) Cyclin D3 sensitizes tumor cells to TNF-induced, c-Myc-dependent apoptosis. Mol. Cell. Biol. 16: 5245 – 5253

Janicke RU, Walker PA, Lin XY and Porter AG (1996) Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. EMBO J. 15: 6969 – 6978

King KL and Cidlowski JA (1995) Cell cycle and apoptosis: common pathways to life and death. J. Cell. Biochem. 58: 175 – 180

Kranenburg O, van der Eb AJ and Zantema A (1996) Cyclin D1 is an essential mediator of neuronal cell death. EMBO J. 15: 46 – 54

Kumar S and Lavin MF (1996) The ICE family of cysteine proteases as effectors of cell death. Cell Death Differ. 3: 255 – 267

Lahti JM, Xiang J, Heath LS, Campana D and Kidd VJ (1995) PITSLRE protein kinase activity is associated with apoptosis. Mol. Cell. Biol. 15: 1 – 11

Lam EW and La Thangue NB (1994) DP and E2F proteins: coordinating transcription with cell cycle progression. Curr. Opin. Cell Biol. 6: 859 – 866

Lam EW-F and Watson RJ (1993) An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. 12: 2705 – 2713

Lee E-H. Chang C-Y, Hu N, Wang Y-C, Lai C-C, Herrup K, Lee W-H and Bradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359: 288 – 294

Lee MH, Williams BO, Mulligan G, Mukai S, Bronson RT, Kyson N, Harlow E and Jacks T (1996) Targeted disruption of p107: functional overlap between p107 and Rb. Genes Dev. 16: 1621 – 1632

Levine A (1997) p53, the cellular gatekeeper for growth and division. Cell 88: 323 – 331

Li CJ, Friedman DJ, Wang C, Metelev V and Pardee AB (1995) Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. Science 268: 429 – 431

Lindahl T, Satoh MS, Poirier GG and Klungland A (1995) Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem. Sci. 20: 405 – 411

Lundgren K, Walwork N, Booher R, Bembski M, Kirshner M and Beach D (1991) mik1 and wee1 cooperate in the inhibitory tryosine phosphorylation of cdc2. Cell 64: 111 – 121

MacLachlan TK, Sang N and Giordano A (1995) Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. Crit. Rev. Eukaryot. Gene Expr. 5: 127 – 156

Martin SJ, McGahon AJ, Nishioka WK, LaFace D, Guo X, Th'ng J, Bradbury EM and Green DR (1995) p34cdc2 and apoptosis. Science 269: 106 – 107

Meikrantz W, Gisselbrecht S, Tam SW and Schlegel R (1994) Activation of cyclin A-dependent protein kinases during apoptosis. Proc. Natl. Acad. Sci. USA 91: 3754 – 3758

Milner J, Cook A and Mason J (1990) p53 is associated with p34cdc-2 in transformed cells. EMBO J. 9: 2885 – 2889

Moran E (1993) Interaction of adenoviral proteins with pRB and p53. FASEB J. 7: 880 – 885

Morgenbesser SD, Williams BO, Jacks T and DePinho RA (1994) p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 371: 72 – 74

Muller R (1995) Transcriptional regulation during the mammalian cell cycle. Trends Genet. 11: 173 – 178

Naik P, Karrim J and Hanahan D (1996) The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors. Genes Dev. 10: 2105 – 2116

Nasmyth K (1996) Viewpoint: putting the cell cycle in order. Science 274: 1643 – 1645

Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R and Pavelka M (1994) Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. J. Cell. Biol. 126: 827 – 837

Ongkeko W, Ferguson DJP, Harris AL and Norbury C (1995) Inactivation of Cdc2 increase the level of apoptosis induced by DNA damage. J. Cell Sci. 108: 2897 – 2904

Paggi MG, Baldi A, Bonetto F and Giordano A (1996) Retinoblastoma protein family in cell cycle and cancer: A review. J. Cell. Biochem. 62: 418 – 430

Peeper DS, Upton TM, Ladha MH, EN, Zalvide J, Bernards R, DeCaprio JA and Ewen ME (1997) Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature 386: 177 – 181

Qin X-Q, Livingston DM, Kaelin WGJ and Adams PD (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. USA 91: 10918 – 10922

Rudolph B, Saffrich R, Zwicker J, Henglein B, Muller R, Ansorge W and Eilers M (1996) Activation of cyclin-dependent kinases by Myc mediates induction of cyclin A, but not apoptosis. EMBO J. 15: 3065 – 3076

Russel P and Nurse P (1987) Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell 49: 559 – 567

Scheffner M, Werness BA, Huibregtse JM, Levine AJ and Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63: 1129 – 1136

Shan B and Lee W-H (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Bio. 14: 8166 – 8173

Sherr CJ (1996) Cancer cell cycles. Science 274: 1672 – 1677

Sherr CJ (1995) D-type cyclins. Trends Biochem. Sci. 20: 187 – 190

Shi L, Nishioka WK, Th'ng J, Bradbury EM, Litchfield DW and Greenberg AH (1994) Premature p34cdc2 activation required for apoptosis. Science 263: 1143 – 1145

Shimizu T, O'Conner PM, Kohn KW and Pommier Y (1995) Unscheduled activation of cyclin B1/Cdc2 kinase in human promyelocytic leukemia cell line HL60 cells undergoing apoptosis induced by DNA damage. Cancer Res. 15: 228 – 231

Sofer-Levi Y and Resnitzky D (1996) Apoptosis induced by ectopic expression of cyclin D1 by not cyclin E. Oncogene 13: 2431 – 2437

Sturzbecher HW, Maimets T, Chumakov P, Brain R, Addison C, Simanis V, Rudge K, Philp R, Grimaldi M, Court W and Jenkins JR (1990) p53 interacts with p34cdc-2 in mammalian cells: implications for cell cycle control and oncogenesis. Oncogene 5: 795 – 801

Symonds H, Krall L, Remington L, Saenz-Robles M, Lowe S, Jacks T and Van Dyke T (1994) p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 78: 703 – 711

Taya Y (1997) RB kinases and RB-binding proteins: new points of view. Trends Biochem. Sci. 22: 14 – 17

Th'ng JP, Wright PS, Hamaguchi J, Lee MG, Norbury CJ, Nurse P and Bradbury EM (1990) The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive CDC2 gene product. Cell 63: 313 – 324

Vousden K (1993) Interactions of human papillomavirus transforming proteins with the products of tumor suppressor genes. FASEB J. 7: 872 – 879

Wang Z-Q, Auer B, Stingl L, Berghammer H, Haidacher D, Schweiger M and Wagner EF (1995) Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. Genes Dev. 9: 509 – 520

Weinberg RA (1995) The retinoblastoma protein and the cell cycle. Cell 81: 323 – 330

Weintraub SJ, Chow KN, Luo RX, Zhang SH, He S and Dean DC (1995) Mechanism of active transcriptional repression by the retinoblastoma protein. Nature 375: 812 – 815

Weintraub SJ, Prater CA and Dean DC (1992) Retinoblastoma protein switches the E2F site from positive to negative element. Nature 358: 259 – 261

White E, Cipriani R, Sabbatini P and Denton A (1991) Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. J. Virol. 65: 2968 – 2978

Williams BO, Remington L, Albert DM, Mukai S, Bronson RT and Jacks T (1994) Cooperative tumorigenic effects of germline mutations in Rb and p53. Nature Genet. 7: 480 – 484

Wu X and Levine AJ (1994) p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA 91: 3602 – 3606

Xiao ZX, Chen J, Levine AJ, Modjtahedi N, Xing J, Sellers WR and Livingston DM (1995) Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature 375: 694 – 698

Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E and Dyson NJ (1996) Tumor induction and tissue atrophy in mice lacking E2F-1. Cell 85: 537 – 548

Yonish-Rouach E, Grunwald D, Wilder S, Kimchi A, May EJ-JL, May P and Oren M (1993) p53-mediated cell death: relationship to cell cycle control. Mol. Cell. Biol. 13: 1415 – 1423

Zhang H, Hannon GJ and Beach D (1994) p21-containing cyclin kinases exist in both active and inactive states. Genes Dev. 81: 1750 – 1758