Negative control elements of the cell cycle in human tumors

Peter D Adams* and William G Kaelin Jr†

The retinoblastoma protein and p53 are both cell-cycle regulators and are, directly or indirectly, inactivated in the majority of human tumors. Recent studies have provided new mechanistic insights into how these proteins regulate cell growth in response to various intracellular and extracellular signals.

Addresses
*Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19104, USA; e-mail: peter_adams@dfci.harvard.edu
†Dana-Farber Cancer Institute and Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA;
e-mail: william_kaelin@dfci.harvard.edu

Current Opinion in Cell Biology 1998, **10**:791–797

http://biomednet.com/elecref/0955067401000791

 ISSN 0955-0674

Abbreviations

APC	anaphase promoting complex/cyclosome
ATM	ataxia telangiectasia mutated
ATR	ATM related
Cdk	cyclin-dependent kinase
HDAC	histone deactylase
HPV	human papilloma virus
RB	retinoblastoma
TSA	trichostatin A

Introduction

The neoplastic phenotype arises, at least in part, as a result of deregulated mitotic cell cycle control. Functional inactivation of negative regulators of the cell cycle will contribute to deregulated cell cycle control and, thereby, to cellular transformation. Two gene products, the retinoblastoma (RB) protein and p53 tumor suppressor proteins, have served as paradigms for the study of negative regulators of the cell cycle [1–4]. These proteins are termed 'tumor suppressors' because their functional inactivation (often through genetic mutation) contributes to the development of cancer in a variety of different tissues. A number of lessons have been learned from their study. Firstly, each of these tumor suppressor proteins is a component of a signal transduction pathway (see Figure 1). Other components of these signalling pathways are also mutated in human cancers, giving rise to the notion of the 'pRB pathway' and the 'p53 pathway'. Inactivation of both pathways is a common, and possibly universal, event during human carcinogenesis. Secondly, pRB and p53 control cellular proliferation in response to distinct events. The pRB pathway responds, in large part, to the presence or absence of mitogenic signals whereas the p53 pathway responds to genotoxic insults and the activation of certain oncogenes. Thirdly, pRB and p53 are both multifunctional and, in addition to regulating the cell cycle, also regulate other processes that affect cell proliferation, such as apoptosis and differentiation. Indeed, the reason why these proteins are so commonly mutated in human cancer may be because they are integrators of diverse signals that ultimately govern the ability of cells to grow in a proper temporally and spatially controlled manner. Examples of other tumor suppressor pathways that regulate the cell cycle are also emerging (see below).

The retinoblastoma protein pathway

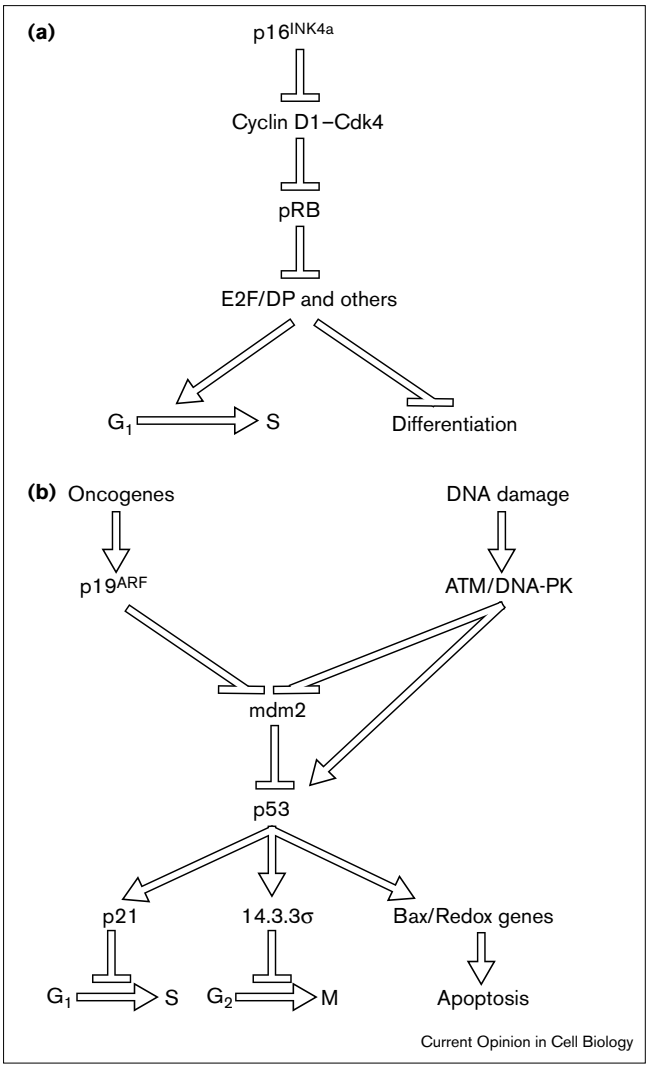
Individuals that inherit a mutant copy of the retinoblastoma tumor suppressor gene (RB) are predisposed to the development of childhood retinoblastoma and, in later life, osteosarcoma. Functional inactivation of pRB is also associated with a number of other sporadic malignancies [5]. pRB plays out its role as a tumor suppressor through its ability to inhibit the mitotic cell cycle, specifically in late G_1 phase, and also to promote terminal differentiation. pRB inhibits progression through late G_1 phase, at least in part, by binding to E2F transcription family members, thereby repressing the transcription of E2F target genes. Progressive phosphorylation of pRB in mid to late G_1 phase by one or more cyclin-dependent kinases (Cdks) (cyclin D1/2/3–cdk4, cyclin E–cdk2 and cyclin A–cdk2) causes dissociation of pRB–E2F complexes, thus allowing expression of E2F target genes and entry into S phase. The activity of the cdks is, in turn, regulated through a number of mechanisms including post-translational modification and complex formation with proteins known as Cdk inhibitors (CDKIs). These include the p21-like CDKIs $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$ and the p16-like CDKIs $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$.

Cyclin D1–cdk4 kinase activity is elevated in a number of different human tumors either through overproduction of cyclin D1 or through mutation of cdk4 so as to make it insensitive to the inhibitory effects of $p16^{INK4a}$. Furthermore, $p16^{INK4a}$ is itself a tumor suppressor protein and is commonly mutated in certain cancers. This pathway of oncogenes and tumor suppressors has been termed the pRB pathway [5] (see Figure 1a). A number of key advances have recently been made in our understanding of this pathway.

The retinoblastoma protein crystal structure

The pRB 'pocket', comprised of subdomains A and B and the intervening spacer, is thought to be the major functional domain of pRB. An intact pocket, as well as much of the adjacent carboxyl terminus, is needed for pRB to bind E2F. Pavletich and coworkers solved the X-ray crystallographic structure of subdomains A and B complexed to the LxCxE (in the single letter code for amino acids, where x is any amino acid)-containing peptide of the human papilloma virus (HPV) E7 oncoprotein [6••]. The LxCxE motif is conserved in a number of different viral oncoproteins, where it is required for binding to pRB and cellular transformation, and in several cellular pRB-binding proteins. Consistent with the crystal structure of the isolated subdomain A and the predictions of Kouzarides and coworkers, each of the A

Figure 1



(a) The pRB pathway regulates the G_1/S transition and differentiation. The figure illustrates the cell cycle control proteins that form the 'core' of the pRB pathway that is functionally inactivated in many, if not all, human cancers. Inactivation of the pathway commonly results from perturbation of p16INK4a, Cyclin D1, Cdk4 or pRB. E2F family members bind to DNA as heterodimers with DP1 or DP2. Of note, E2F/DP family members are not commonly mutated in human cancers, suggesting that there is significant divergence of the pathway downstream of pRB and/or that other key pRB effector proteins, that are commonly mutated in human cancer, remain to be identified. These possibilities are indicated by 'others'. **(b)** The p53 pathway regulates cell cycle progression and apoptosis. The figure illustrates the upstream pathways that are responsible for activation of p53 in response to DNA damage and activation of oncogenes and some of the downstream genes that mediate the p53 dependent G_1/S and G_2/M arrests and apoptosis. In addition to p53 itself, a number of the upstream regulators, such as mdm2, p19ARF, the oncogenes responsible for activation of p19ARF (see text) and ATM, are mutated in human cancer. Due to space limitations, induction of apoptosis by Bax and the 'Redox genes' are not discussed further in the text (see [66••] and references therein for further details).

and B subdomains constitutes a five-helix 'cyclin fold', of the type previously identified in cyclin A and the basal transcription factor TFIIB [7,8]. The LxCxE peptide of HPV E7 binds within a shallow groove of the cyclin fold of subdomain B. The amino acids lining this groove are among the most highly conserved between pRB of different species and the related proteins, p107 and p130. A second cluster of highly conserved amino acids is present at the interface between subdomains A and B and this is proposed to be the binding site for E2F family members. This is consistent with earlier mutagenesis studies which showed that E2Fs bind to a site on pRB which is distinct from the site which interacts with LxCxE-containing proteins [9,10]. Potentially noteworthy in this regard is the finding that the pRB A and B domains, when produced as separate polypeptides, can form dimers that reconstitute an active pRB pocket [11]. A number of naturally occurring, inactivating point mutations in pRB map to subdomain B and the interface between subdomains A and B.

Different cyclin-dependent kinases phosphorylate the retinoblastoma protein on different sites

A number of different cdks are active at the time in the cell cycle when pRB is progressively phosphorylated. These include cyclin D1/2/3–cdk4, cyclin E–cdk2 and cyclin A–cdk2. Moreover, pRB is phosphorylated on multiple sites *in vivo* and a number of studies have demonstrated that different cdks preferentially phosphorylate pRB on distinct sites. For example, cyclin A–cdk2 preferentially phosphorylates Thr821 and cyclin D–cdk4 Ser780 and Ser795 [12,13••,14••]. These results, together with those of Knudsen *et al.* which suggest that phosphorylation of pRB on different sites regulates binding to distinct effector proteins [15], raise the possibility that different Cdks might regulate different pRB functions. Consistent with this notion, Lundberg *et al.* showed that functional inactivation of pRB required phosphorylation by both cdk4 and cdk2 [16•] and Knudsen *et al.* suggested that pRB governs passage through a point in G_1 termed the restriction point and the G_1/S boundary through distinct phosphorylation sensitive mechanisms [17•].

Transcriptional repression by retinoblastoma protein–E2F and HDAC1

When bound to DNA, E2F–pRB complexes function as active repressors of transcription [18]. A number of lines of evidence suggest that relief of this repression is responsible for the timely activation of E2F target genes at the G_1/S transition. Histone deacetylases (HDACs) have been shown to mediate the repressor activity of a number of different transcriptional repressors [19]. Three groups showed that pRB binds to HDAC1, suggesting that an E2F–pRB–HDAC1 complex might be responsible for transcriptional repression of E2F target genes [20••–22••]. Consistent with this idea, transcriptional repression by pRB was relieved by the HDAC1 inhibitor trichostatin A (TSA). Luo *et al.*, however, noted that the ability of TSA to negate transcriptional repression by pRB was promoter dependent [22••]. This suggests, that pRB might employ HDAC-independent mechanisms to repress transcription as well. In this

regard, it was earlier shown that pRB could bind to adjacent transcriptional activation domains when tethered to DNA and can also affect DNA bending [23, 24].

Retinoblastoma protein and induction of differentiation

The pRB is required for differentiation in the developing mouse embryo and in a number of *in vitro* differentiation systems [25]. For example, pRB is required for *in vitro* differentiation of adipocytes and muscle. Adipocyte differentiation appears to involve a direct interaction between pRB and the transcription factor, CCAAT enhancer binding protein (C/EBP) [26], and muscle differentiation requires a potentiation of activity of the muscle specific transcription factor, MyoD, in a manner that does not require a physical interaction [27–29]. One possibility, among several, is that pRB serves to sequestor a cellular inhibitor of differentation. Another likely regulator of pRB-mediated differentiation is HBP-1 [30]. HBP-1 contains two consensus pRB-binding motifs and is upregulated during differentation. One function of HBP-1 may be to transcriptionally silence the N-myc gene upon cell cycle withdrawal.

The relationship between pRB-mediated differentiation and cell cycle control, and the relative contributions of these two processes to tumor suppression by pRB, has been unclear. Sellers *et al.* [31•] identified pRB pocket mutants that were able to promote differentiation, but were unable to induce an acute G_1/S block following their reintroduction into pRB-defective tumor cells. In these assays, the ability of pRB to induce a G_1/S growth arrest correlated with its ability to bind to E2F and repress transcription, whereas induction of differentiation did not. Importantly, two pRB mutants, pRBΔ4 and pRBTry661, that are associated with a low risk of cancer were, like the products of high risk, null RB alleles, unable to bind to E2F and consequently were unable to induce an acute G_1/S block. Unlike the high risk mutants, however, they did retain the ability to promote differentiation. This suggests that cell cycle control and differentiation are separable functions of pRB and that both contribute independently to tumor suppression.

The p53 pathway

Inheritance of a mutant allele of the p53 tumor suppressor gene is the cause of Li-Fraumeni syndrome which is characterized by predisposition to a range of cancers. Functional inactivation of p53 is also associated with sporadic tumors [2]. The tumor suppressor activity of p53 stems from its ability to both inhibit the mitotic cell cycle and promote apoptosis (Figure 1b). In response to DNA damage or activation of oncogenes, the p53 protein is post-translationally stabilized and can consequently initiate cell cycle arrest at either the G_1/S or G_2/M transitions. Induction of a G_1/S block by p53 is due, at least partly, to its ability to transcriptionally activate the $p21^{CIP1}$ gene. The resulting elevated levels of $p21^{CIP1}$ protein inhibit the activity of G_1 Cdks, thereby bringing about a growth arrest in late G_1. Until recently, relatively little was known concerning the basis for the p53 dependent G_2/M cell cycle arrest and mechanisms of activation of p53.

Regulation of p53 stability by Mdm2

Activation of p53 by oncogenes or DNA damage is associated with post-translational stabilization of the protein. The demonstration that mdm2, a known p53 binding protein and an inhibitor of p53-dependent transactivation, is able to promote the degradation of p53 suggested that stabilization of p53 might result, at least in part, from inhibition of mdm2 activity [32••,33••]. Support for this notion has subsequently come from other studies (see below).

Activation of p53 by oncogenes

Certain oncogenes, including c-myc, E2F1, and E1A, stimulate p53-dependent apoptosis [34–38]. Recently, it was shown that such oncogenes increase expression of $p19^{ARF}$ (a protein product derived from an overlapping but an alternative reading frame of $p16^{INK4a}$) [39], thereby initiating a cascade of events that culminates in the stabilization and activation of p53 [40••–43••] (Figure 1b). Activation of p53 in this setting is strictly dependent upon $p19^{ARF}$ whereas inhibition of cell growth by $p19^{ARF}$ requires p53 [44••]. How $p19^{ARF}$ activates p53 is not precisely clear but is likely to involve its ability to bind to mdm2 and, under certain conditions, to p53 itself [45]. In the simplest model, $p19^{ARF}$ prevents mdm2 from targeting p53 for degradation and from masking the p53 transactivation domain.

Activation of p53 by DNA damage

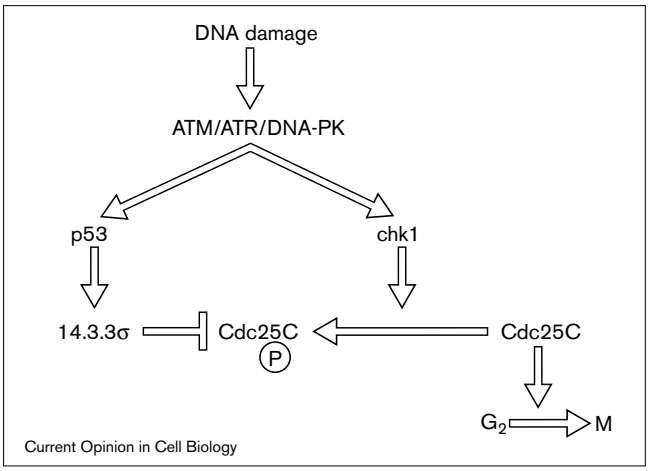
Stabilization of p53 in response to DNA damage does not require p19ARF but instead involves the ataxia telangiectasia mutated (ATM) protein kinase [44••,46]. The ATM gene is mutated in the hereditary cancer syndrome ataxia telangiectasia [47]. In response to DNA damage, the amino terminus of p53 becomes phosphorylated [48••,49••]. In $ATM^{-/-}$ cells, phosphorylation and stabilization of p53 in response to ionizing radiation are delayed [46,48••]. Conceivably, phosphorylation of p53 and/or mdm2 prevents the degradation of p53 by mdm2.

A protein related to ATM, DNA-dependent protein kinase (DNA-PK), phosphorylates p53 on a site, Ser15, that is phosphorylated *in vivo* in response to DNA damage [48••,49••]. Moreover, DNA-PK is required for the DNA-damage-induced increase in p53 DNA-binding and transcriptional activity [50••]. Interestingly, stabilization of p53 appears to be normal in $DNA\text{-}PK^{-/-}$ cells. Thus, it seems likely that in response to DNA damage the kinases ATM and DNA-PK, and perhaps others, cooperate to fully stabilize and activate p53 (Figure 1b).

The p53 dependent G_2/M arrest

Hermeking *et al.* identified the protein 14.3.3σ as a transcriptional target gene of p53 that is likely to mediate, at least in part, the p53-dependent G_2/M arrest in response to DNA damage [51••]. The ability of 14.3.3σ to induce a G_2/M cell cycle arrest is due to its ability to bind to, and probably sequester in the cytoplasm, the cdc25C phosphatase, an activator of the mitotic kinase cyclin B–cdc2 [52•–54•]. Interestingly, 14.3.3 proteins bind only to

Figure 2



Coordination of the DNA damage dependent G_2/M arrest. Activation of the ATM and/or ATM related kinase (ATR) by DNA damage results, directly or indirectly, in activation of p53 and the kinase chk1. p53 transcriptionally activates 14.3.3σ and chk1 phosphorylates cdc25C on Ser216. Binding of 14.3.3σ proteins to phosphorylated cdc25C results in functional inactivation of cdc25C, through a mechanism that is not entirely clear but which may involve sequestration in the cytoplasm. Inactivation of cdc25C inhibits the G_2/M transition by preventing the dephosphorylation and activation of cdc2–cyclin B.

cdc25C that is phosphorylated on Ser216 by the DNA-damage-activated kinase, chk1 (Figure 2).

In *Schizosaccharomyces pombe* chk1 is activated by DNA damage in a manner dependent upon the checkpoint gene, Rad3 [55]. Rad3 is homologous to the ATM kinase and even more closely related to another kinase, ATM related (ATR) [56,57]. Since ATM or ATR might also be partly responsible for the DNA damage dependent activation of p53 (see above), DNA damage might induce a G_2/M arrest through the coordinated chk1-dependent phosphorylation of cdc25C and the p53-dependent increase in expression of 14.3.3σ, an inhibitor of phosphorylated cdc25C (Figure 2).

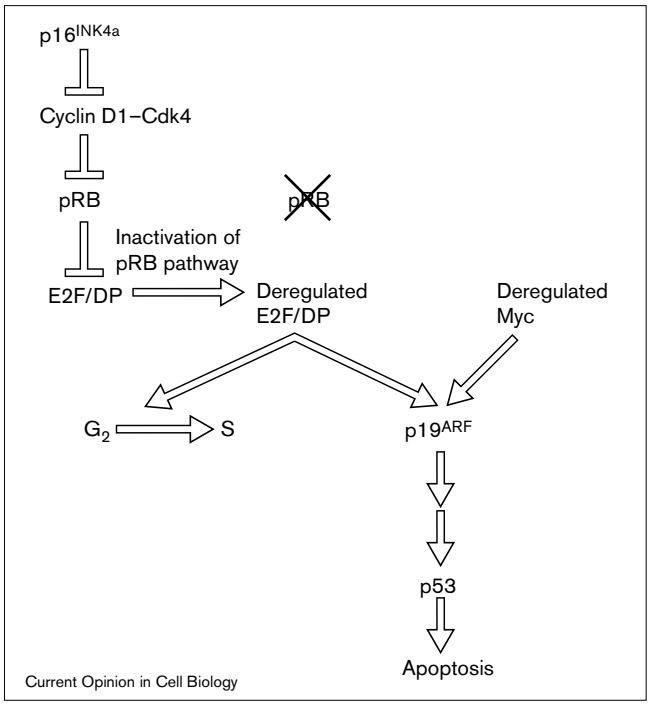
Novel p53 family members

Two mammalian proteins, p73 and p51, with significant sequence homology to p53 have recently been identified [58••–61••]. Both p73 and p51 can bind to canonical p53 DNA binding sites, activate transcription and induce apoptosis when overproduced. Preliminary data suggest that these proteins, unlike p53, are rarely mutated in human cancers. This observation suggests that p51 and p73, under physiological conditions, cannot fully compensate for p53 loss in the tissues examined to date. Alternatively, these genes may be silenced epigenetically.

Interactions between the retinoblastoma protein and p53 pathways

The pRB and p53 pathways interact in at least three ways. Firstly, the p53 dependent G_1/S cell cycle arrest is likely to result, at least in part, from the transcriptional induction of

Figure 3



Interactions between the pRB and p53 pathways. Functional inactivation of the pRB pathway results in deregulated E2F/DP activity. This has been known for a number of years to result in stimulation of DNA synthesis and p53 dependent apoptosis. It seems that p53 dependent apoptosis results from activation of $p19^{ARF}$ which, indirectly, activates p53. p53-dependent Myc induced apoptosis also depends upon the $p19^{ARF}$ pathway.

$p21^{CIP1}$ and the consequent inhibition of the G_1 Cdks. Inhibition of the G_1 Cdks converts pRB into the hypophosphorylated growth suppressive form. It is important to note, however, that induction of a G_1/S block by p21-like CDKIs, in contrast to p16-like CDKIs, does not require the presence of functional pRB.

Secondly, the p53 pathway protects against deregulation of the pRB pathway (Figure 3). Specifically, functional inactivation of pRB results in deregulated E2F activity which, in turn, results in induction of $p19^{ARF}$ and p53-dependent apoptosis [36,40••]. This may explain why many human tumors in which the pRB pathway is altered also contain mutations affecting the p53 pathway.

Thirdly, and very perplexingly, a single genetic locus encodes two different proteins, $p16^{INK4a}$ and $p19^{ARF}$, that negatively regulate the pRB and p53 pathways respectively. In view of the importance of the pRB and p53 pathways to cell growth control, such an arrangement would appear to be disadvantageous. The importance of this locus has been established using mouse knockouts. Mice lacking only $p19^{ARF}$ develop tumors at high frequency and the spectrum of tumors is similar to that previously ascribed to loss of $p16^{INK4a}$ [44••,62]. Whether the tumors originally

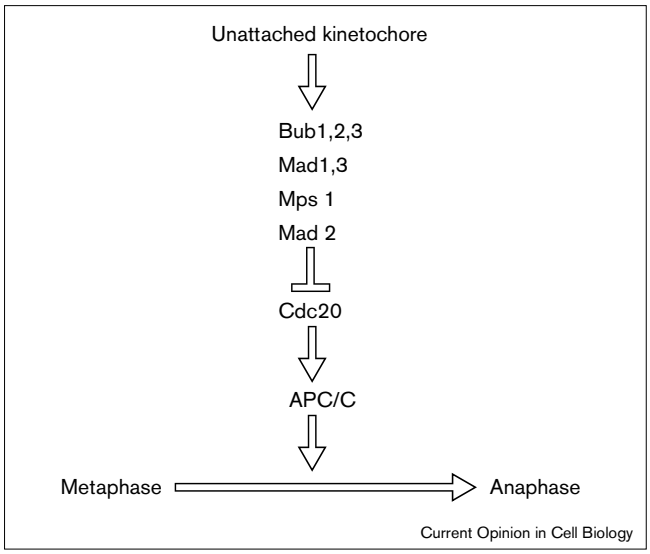
described in $p16^{INK4a}$ knockout mice were actually due to inadvertent disruption of $p19^{ARF}$ or whether loss of $p19^{ARF}$ and loss of $p16^{INK4a}$ give rise to similar phenotypes is an issue that remains to be resolved.

The spindle assembly checkpoint pathway

The premature onset of mitotic anaphase prior to the proper attachment of all of the chromosomes to the mitotic spindle would, presumably, result in the missegregation of chromosomes between the daughter cells. Such missegregation might be lethal or contribute to genomic instability and the genesis of cancer. Therefore, a checkpoint exists to ensure that all of the chromosomes are properly attached to the mitotic spindle before the onset of anaphase [63].

Similar to the pRB and p53 pathways, this checkpoint appears to consist of a number of gene products acting in a linear pathway. In yeast, in the presence of an unattached kinetochore, the Mad, Bub and Mps1 proteins act to block the onset of anaphase by inhibition of the anaphase promoting complex/cytosome (APC/C) (Figure 4). Recently, the human homologue of at least one of these proteins, hsBUB1, has been shown to be mutated in two colorectal tumors [64••]. Furthermore, the hsMAD1 protein may be a cellular target of the human T-cell leukemia virus 1 (HTLV-1) oncoprotein, Tax [65••]. Thus, perturbation or mutation of the checkpoint elements that negatively regulate the onset of anaphase might contribute to the development of cancer.

Figure 4



The mitotic spindle assembly checkpoint pathway. A kinetochore that is not attached to the mitotic spindle initiates a signal transduction pathway that culminates in the inhibition of the APC and a block to the separation of sister chromatids. In *Saccharomyces cerevisiae*, the inhibition of the APC depends upon the Bub, Mad and Mps1 gene products. The human homologue of one of these proteins, hsBUB1, is mutated in colorectal tumours and the hsMAD1 protein may be a cellular target of the HTLV-1 oncoprotein, Tax. Functional inactivation of the mitotic spindle assembly pathway might promote missegregation of chromosomes, genomic instability and cancer.

Conclusion

This review has emphasized the notion that both the pRB and p53 tumor suppressor proteins both fall within signal transduction pathways that each consist of a number of oncogenes and tumor suppressor genes. We have also highlighted the possibility of at least one other such pathway, the spindle assembly checkpoint pathway that is operative at the metaphase-anaphase transition. Major questions for the future are to determine how other known tumor suppressor proteins, such as BRCA1 and WT1, fit into these or similar pathways and to determine why some components of these pathways, such as $p21^{CIP1}$ in the pRB pathway, are not commonly mutated in human cancer.

It has been apparent for some time that pRB is a multifunctional protein and probably binds to multiple effector proteins simultaneously. It seems likely that different pRB binding proteins mediate different pRB functions, such as induction of differentiation and the block to entry into S phase. A major challenge is to dissect the causal relationships between pRB biological activities, pRB effector proteins and the multiple cdks.

Much progress has been made recently in our understanding of the p53 pathway. Many questions remain, however. For example, how does $p19^{ARF}$ discriminate between physiological and pathological activation of proto-oncogenes? How exactly do ATM, DNA-PK and other kinases cooperate to activate p53 and how are they themselves activated?

For both p53 and pRB, what are the relative contributions of their different biological activities, for example induction of cell cycle arrest and apoptosis in the case of p53, to the their tumor suppression function? Finally, might dysregulation of mitotic cell cycle control be exploited to treat cancer in humans?

Acknowledgements

We thank all members of the Kaelin, Sellers, Ewen, DeCaprio and Livingston laboratories for much stimulating discussion.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Planas-Silva MD, Weinberg RA: **The restriction point and control of cell proliferation.** *Curr Opin Cell Biol* 1997, **9**:768-772.
2. Gottlieb TM, Oren M: **p53 in growth control and neoplasia.** *Biochim Biophys Acta* 1996, **1287**:77-102.
3. Ko LJ, Prives C: **p53: puzzle and paradigm.** *Genes Dev* 1996, **10**:1054-1072.
4. Taya Y: **RB kinases and RB-binding proteins: new points of view.** *Trends Biochem Sci* 1997, **22**:14-17.

5. Sellers WR, Kaelin WG Jr: **Role of the retinoblastoma protein in the pathogenesis of human cancer.** *J Clin Oncol* 1997, **15**:3301-3312.

6. •• Lee J-O, Russo AA, Pavletich NP: **Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7.** *Nature* 1998, **391**:859-865.

This paper provides structural insight into a number of aspects of pRB function and its inactivation through phosphorylation, mutation and binding of viral oncoproteins. It also pin-points the interaction site for LxCxE proteins and suggests a second site for E2F family members. Finally, it places pRB firmly in the camp of proteins that contain cyclin folds that function as protein–protein interaction domains, along with cyclin A and TFIIB.

7. Kim HY, Cho Y: **Structural similarity between the pocket region of retinoblastoma tumour suppressor and the cyclin-box.** *Nature Struct Biol* 1997, **4**:390-395.

8. Gibson TJ, Thompson JD, Blocker A, Kouzarides T: **Evidence for a protein domain superfamily shared by the cyclins, TFIIB and pRB/p107.** *Nucleic Acids Res* 1994, **22**:946-952.

9. Wu EW, Clemens KE, Heck DV, Munger K: **The human papillomavirus E7 oncoprotein and the cellular transcription factor E2F bind to separate sites on the retinoblastoma tumor suppressor protein.** *J Virol* 1993, **67**:2402-2407.

10. Ikeda M-A, Nevins J: **Identification of distinct roles for separate E1A domains in disruption of E2F complexes.** *Mol Cell Biol* 1993, **13**:7029-7035.

11. Chow KN, Dean DC: **Domains A and B in the Rb pocket interact to form a transcriptional repressor motif.** *Mol Cell Biol* 1996, **16**:4862-4868.

12. Kitagawa M, Higashi H, Jung H-K, Suzuki-Takahashi I, Ikeda M, Tamai K, Kato J, Seqawa K, Yoshida E, Nishimura S, Taya Y: **The consensus motif for phosphorylation by cyclin D1-cdk4 is different from that for phosphorylation by cyclin A/E-cdk2.** *EMBO J* 1996, **15**:7060-7069.

13. •• Connell-Crowley L, Harper JW, Goodrich DW: **Cyclin D1/cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation.** *Mol Biol Cell* 1997, **8**:287-301.

These papers [13••,14••] along with [12,15] provide evidence to support the notion that different cyclin-dependent kinases phosphorylate different sites on pRB and that phosphorylation of pRB on different sites modulates distinct functions. It follows from this that different cyclin-dependent kinases regulate distinct pRB dependent functions. A clearer understanding of such ideas is crucial to a full understanding of the mechanism by which cells traverse the restriction point and the G_1/S transition..

14. •• Zarkowska T, Mittnacht S: **Differential phosphorylation of the retinoblastoma protein by G_1/S cyclin-dependent kinases.** *J Biol Chem* 1997, **272**:12738-12746.

See annotation to [13••].

15. Knudsen ES, Wang JY: **Differential regulation of retinoblastoma function by specific cdk phosphorylation sites.** *J Biol Chem* 1996, **271**:8313-20.

16. • Lundberg AS, Weinberg RA: **Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin/cdk complexes.** *Mol Cell Biol* 1998, **18**:753-761.

These two papers [16•,17•] provide biological support for the notion that different aspects of pRB function are regulated by distinct phosphorylation events.

17. • Knudsen ES, Buckmaster C, Chen TT, Feramisco JR, Wang JY: **Inhibition of DNA synthesis by RB: effects on G_1/S transition and S-phase progression.** *Genes Dev* 1998, **12**:2278-2292.

See annotation to [16•].

18. Weintraub SJ, Prater CA, Dean DC: **Retinoblastoma protein switches the E2F site from positive to negative element.** *Nature* 1992, **358**:259-261.

19. Kuo M-H, Allis CD: **Roles of histone acetyltransferases and deacetylases in gene regulation.** *Bioessays* 1998, **20**:615-626.

20. •• Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T: **Retinoblastoma protein recruits histone deacetylase to repress transcription.** *Nature* 1998, **391**:597-601.

Together these three papers [20••–22••] suggest that at least one mechanism by which pRB–E2F complexes mediate transcriptional repression is through binding to a histone deacetylase activity. HDAC1 is shown to contain a functional LxCxE (in the single letter code for amino acids where x is any amino acid) motif, suggesting that it may be a target of the transforming viral oncoproteins such as SV40 T antigen, HPV E7 and Adenovirus E1a.

21. •• Magnaghi-Jaulin L, Groisman R, Naguibneva I, Robin P, Lorain S, Le Villain JP, Troalen F, Trouche D, Harel-Bellan A: **Retinoblastoma protein represses transcription by recruiting a histone deacetylase.** *Nature* 1998, **391**:601-605.

See annotation to [20••].

22. •• Luo RX, Postigio AA, Dean DC: **Rb interacts with histone deacetylase to repress transcription.** *Cell* 1998, **92**:463-473.

See annotation to [20••].

23. Cress WD, Nevins JR: **A role for bent DNA structure in E2F-mediated transcription activation.** *Mol Cell Biol* 1996, **16**:2119-2127.

24. Huber HE, Goodhart PJ, Huang PS: **Retinoblastoma protein reverses DNA bending by transcription factor E2F.** *J Biol Chem* 1994, **269**:6999-7005.

25. Yee AS, Shih HH, Tevosian SG: **New perspectives on retinoblastoma family functions in differentiation.** *Front Biosci* 1998, **3**:532-547.

26. Chen PL, Riley DJ, Chen Y, Lee WH: **Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs.** *Genes Dev* 1996, **10**:2794-2804.

27. Novitch BG, Mulligan GJ, Jacks T, Lassar AB: **Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G_2 phases of the cell cycle.** *J Cell Biol* 1996, **135**:441-456.

28. Gu W, Schneider JW, Condorelli G, Kaushal S, Mahdavi V, Nadal-Ginard B: **Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation.** *Cell* 1993, **72**:309-324.

29. Zacksenhaus E, Jiang Z, Chung D, Marth JD, Phillips RA, Gallie BL: **pRB controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis.** *Genes Dev* 1996, **10**:3051-3064.

30. Tevosian SG, Shih H, Mendelson KG, Sheppard KA, Paulson KE, Yee AS: **HBP-1: an HMG box transcriptional repressor that is targetted by the retinoblastoma family.** *Genes Dev* 1997, **11**:383-396.

31. • Sellers WR, Novitch BG, Miyake S, Heith A, Otterson GA, Kaye FJ, Lassar AB, Kaelin WGJ Jr: **Stable binding of E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumour cell growth.** *Genes Dev* 1998, **12**:95-106.

This paper provides evidence that the induction of differentiation and cell cycle arrest by pRB are separate events. It follows from this that both pRB induced differentiation and cell cycle arrest might contribute independently to tumor suppression, and evidence for this is provided. It also follows that cell cycle arrest and differentiation are mediated by distinct molecular mechanisms and evidence for this was obtained.

32. •• Kubbutat MHG, Jones SN, Vousden KH: **Regulation of p53 stability by mdm2.** *Nature* 1997, **387**:299-303.

These papers [32••,33••] are significant because they demonstrate that mdm2 is a key determinant of p53 stability. Since p53 activity is thought to be regulated in large part through the stability of the protein, they suggest that the signal transduction pathways that activate p53 in response to DNA damage or activation of cellular oncogenes might feed through mdm2.

33. •• Haupt Y, Maya R, Kazaz A, Oren M: **Mdm2 promotes the rapid degradation of p53.** *Nature* 1997, **387**:296-299.

See annotation to [32••].

34. Lowe SW, Ruley E: **Stabilisation of the tumour suppressor p53 is induced by adenovirus E1a and accompanies apoptosis.** *Genes Dev* 1993, **7**:535-545.

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

36. Wu X, Levine AJ: **p53 and E2F1 cooperate to mediate apoptosis.** *Proc Natl Acad Sci USA* 1994, **91**:3602-3606.

37. Hermeking H, Eick D: **Mediation of c-Myc induced apoptosis by p53.** *Science* 1994, **265**:2091-2093.

38. Wagner AJ, Kokontis JM, Hay N: **Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21WAF1/CIP1.** *Genes Dev* 1994, **8**:2817-2830.

39. Quelle DE, Zindy F, Ashmun RA, Sherr CJ: **Alternative reading frames of the INK4a tumour suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest.** *Cell* 1995, **83**:993-1000.

40. •• de Stanchina E, McCurrach ME, Zindy F, Shieh S-Y, Ferbeyre G, Samuelson AV, Prives C, Roussel M, Sherr CJ, Lowe SW: **E1a signalling to p53 involves the p19ARF tumour suppressor.** *Genes Dev* 1998, **12**:2434-2442.
These papers [40••,41••] confirm the concepts implicit in [42••–44••]. That is, that a signal transduction cascade feeds through p19ARF and mdm2 to activate p53 in response to activation of oncogenes. Interestingly, [44••] showed that the role of p19ARF in activation of p53 is restricted to activation by oncogenes and it is not required for activation by DNA damage. This implies that oncogenes activate p53 in a manner that is independent of any capacity that they have to cause DNA damage.

41. •• Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF: **Myc signalling via the ARF tumour suppressor regulates p53-dependent apoptosis and immortalisation.** *Genes Dev* 1998, **12**:2424-2433.
See annotation to [40••].

42. •• Zhang Y, Xiong Y, Yarborough WG: **ARF promotes MDM2 degradation and stabilises p53: ARF-INK4a locus deletion impairs both the RB and p53 tumour suppression pathways.** *Cell* 1998, **92**:725-734.
These papers [42••,43••] demonstrate that $p19^{ARF}$ is a negative regulator of mdm2. Considered together with [32••,33••] they suggest that a signal transduction pathway including $p19^{ARF}$ and mdm2 might be responsible for stabilization of p53.

43. •• Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee HW *et al.*: **The INK4a tumour suppressor gene product, p19ARF, interacts with MDM2 and neutralises MDM2's inhibition of p53.** *Cell* 1998, **92**:713-723.
See annotation to [42••].

44. •• Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G, Sherr CJ: **Tumour suppression at the mouse INK4a locus mediated by the alternative reading frame product 19ARF.** *Cell* 1997, **91**:649-659.
This paper provided the first clue that $p19^{ARF}$ might function as an upstream activator of p53. Moreover, it also showed that the tumour phenotype of mice lacking $p19^{ARF}$ is very similar to the phenotype that had been previously ascribed to loss of $p16^{INK4a}$ [62]. Thus it raised a question mark over the relative tumour suppressive activities of $p19^{ARF}$ and $p16^{INK4a}$.

45. Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF, Sherr CJ: **Functional and physical interactions of the ARF tumour suppressor with p53 and mdm2.** *Proc Natl Acad Sci USA* 1998, **95**:8292-8297.

46. Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ Jr: **A mammalian cell cycle checkpoint pathway utilising p53 and GADD45 is defective in ataxia-telangiectasia.** *Cell* 1992, **71**:587-597.

47. Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S: **A single ataxia-telangiectasia gene with a product similar to PI-3 kinase.** *Science* 1995, **268**:1749-1753.

48. •• Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan M: **DNA damage induces phosphorylation of the amino-terminus of p53.** *Genes Dev* 1997, **11**:3471-3481.
This paper demonstrates that p53 becomes phosphorylated on Ser15 in response to ionizing or UV irradiation. Together with [49••] it suggests that phosphorylation of p53 in response to DNA damage might inhibit degradation promoted by mdm2. This paper also shows that, at least in response to ionizing radiation, phosphorylation of Ser15 is partly dependent upon the ATM kinase.

49. •• Shieh SY, Ikeda m, Taya Y, Prives C: **DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2.** *Cell* 1997, **91**:325-334.
This paper demonstrates that phosphorylation of p53 on Ser15 by DNA-dependent protein kinase (DNA-PK), a kinase related to ATM, results in inhibition of mdm2 binding. Thus, phosphorylation at this site would be expected to both stabilize p53 and promote transactivation. Reference [48••] suggests that ATM is required for efficient stabilization of p53, at least in response to ionizing radiation, and reference [50••] suggests that DNA-PK is required for DNA-binding of and transcriptional activation by p53, but not for stabilization of p53.

50. •• Woo RA, McLure KG, Lees-Miller SP, Rancourt DE, Lee PWK: **DNA -dependent protein kinase acts upstream of p53 in response to DNA damage.** *Nature* 1998, **394**:700-704.
See annotation to [49••].

51. •• Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B: **14-3-3 sigma is a p53-regulated inhibitor of G_2/M progression.** *Mol Cell* 1997, **1**:3-11.
This paper demonstrates a likely molecular basis for the previously identified p53 dependent G_2/M arrest. In proposing a role for 14.3.3 proteins in this checkpoint it is entirely consistent with the recent studies of others [52•–54•]; moreover, since 14.3.3 proteins have homologues in *Schizosaccharomyces pombe* in the guise of the checkpoint genes Rad24 and Rad25, it provides a point of convergence between yeast checkpoint genetics and a p53 dependent checkpoint.

52. • Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H: **Mitotic and G_2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of cdc25C on serine-216.** *Science* 1997, **277**:1501-1505.
See annotation to [51••].

53. • Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, Elledge SJ: **Conservation of the chk1 checkpoint pathway in mammals: linkage of DNA damage to cdk regulation through cdc25.** *Science* 1997, **277**:1497-1501.
See annotation to [51••].

54. • Furnari B, Rhind N, Russell P: **Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase.** *Science* 1997, **277**:1495-1497.
See annotation to [51••].

55. Walworth NC, Bernards R: **Rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint.** *Science* 1996, **271**:353-356.

56. Keegan KS, Holtzman DA, Plug AW, Christenson ER, Brainerd EE, Flaggs G, Bentley NJ, Taylor EM, Meyn MS, Moss SB *et al.*: **The ATR and ATM protein kinases associate with different sites along meiotically pairing chromosomes.** *Genes Dev* 1996, **10**:2423-2437.

57. Zakian VA: **ATM-related genes: what do they tell us about functions of the human gene?** *Cell* 1995, **82**:685-687.

58. •• Kaghad M, Bonnet H, Yang A, Creancier L, Biscan J-C, Valent A, Minty A, Chalon P, Lelias J-M, Dumont X *et al.*: **Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers.** *Cell* 1997, **90**:809-819.
These papers [58••–61••] reveal the existence of the p53-related family members p73 and p51. The existence of these proteins raises important questions regarding their functional similarities and differences with respect to p53.

59. •• Jost CA, Marin MC, Kaelin WG Jr: **p73 is a human p53-related protein that can induce apoptosis.** *Nature* 1997, **389**:191-194.
See annotation to [58••].

60. •• Osada M, Ohba M, Kawahara C, Ishioka C, Kanamaru R, Katoh I, Ikawa Y, Nimura Y, Nakagawara A, Obinata M, Ikawa S: **Cloning and functional analysis of human p51, which structurally and functionally resembles p53.** *Nat Med* 1998, **4**:839-843.
See annotation to [58••].

61. •• Trink B, Okami K, Wu L, Sriuranpong V, Jen J, Sidransky D: **A new human p53 homologue.** *Nat Med* 1998, **4**:747-748.
See annotation to [58••].

62. Serrano M, Lee H-W, Chin L, Cordon-Cardo C, Beach D, DePinho RA: **Role of INK4a locus in tumour suppression and cell mortality.** *Cell* 1996, **85**:27-37.

63. Harwick KG: **The spindle checkpoint.** *Trends Genet* 1998, **14**:1-4.

64. •• Cahill DP, Lengauer C, Yu J, Riggins GJ, Willson JKV, Markowitz SD, Kinzler KW, Vogelstein B: **Mutations of mitotic checkpoint genes in human cancers.** *Nature* 1998, **392**:300-303.
These two papers [64••,65••] are the first to suggest that proteins in the mitotic spindle assembly checkpoint pathway are targets for functional inactivation in human cancer. As such, they further extend the concept of 'tumour suppression pathways' that was initially established through the study of pRB and p53.

65. •• Jin D-Y, Spencer F, Jeang K-T: **Human T cell leukemia virus type 1 oncoprotein tax targets the human mitotic checkpoint protein MAD1.** *Cell* 1998, **93**:81-91.
See annotation to [64••].

66. •• Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B: **A model for p53-induced apoptosis.** *Nature* 1997, **389**:300-305.
This paper describes a powerful technique (SAGE) for isolating differentially expressed mRNAs. Using this approach, the authors show that p53 activates the transcription of redox-related genes culminating in the generation of reactive oxygen species that can degrade mitochondria.