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Integration of the pRB and p53 cell cycle control pathways

Craig L. Stewart[1], Alejandro Moro Soria[1,2] and Paul A. Hamel[1]
[1]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada;* [2]*Centre for Genetic Engineering and Biotechnology, Havana, Cuba*

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Summary

Two fundamental molecular pathways, the pRB and p53 pathways, regulate cell growth and cell death. The importance of these pathways in cellular growth control is underscored by the observation that members of these pathways are found mutated in all human cancers. These two pathways have typically been studied and described independently. However, as we discuss here, recent data have revealed an intimate molecular and genetic interaction between the p53 and pRB pathways.

Progression through or exit from the eukaryotic cell division cycle is regulated by a series of stringent control mechanisms. The cell cycle most commonly depicted (see Figure 1) consists of two major phases, one where replication of genome occurs (S phase) and another responsible for segregation of the duplicated genome into daughter cells (mitosis or M phase). These two phases are typically separated by gaps; G_1 between M and S and G_2, between S and M. While not the subject of this review, variations of this general scheme are employed by many cell types where, for example, the gaps are absent, mitosis proceeds in the absence of DNA synthesis or where a cell completes additional rounds of DNA synthesis without passing through M. It is also clear that the regulation of the cell cycle involves mechanisms which are highly conserved among all eukaryotes. This conservation is particularly evident for mitosis where replacement of defective proteins controlling M in *Saccharomyces cerevisiae* with their human counterparts restores normal mitosis (for review see [1]).

For this review, we restrict consideration of cell cycle to control of the G_0/G_1 to S transition. Cells in G_0 or G_1 can be stimulated by mitogens (growth factors) to progress through G_1 towards S phase. This transition is mitogen-dependent until the cells reach the 'restriction point' [2]. After this point, cells are irreversibly committed to DNA synthesis regardless of the presence of the mitogenic signal. Thus, the restriction point represents a critical checkpoint in the cell cycle. Here, integration of an array of endogenous and exogenous signals leads either to cell cycle arrest or continuation through the cell cycle to mitosis. Due to its irreversible nature, the restriction point is tightly regulated. Passage through this checkpoint is governed by both positive and negative cell cycle regulatory factors. It is also evident that additional checkpoints are important regulators of progression after the restriction point has been passed. These checkpoints ensure the proper timing of specific events in the cell cycle and assess the fidelity of DNA synthesis. When these checkpoints are invoked, cells can be halted from progressing further through the cell cycle until, for example, DNA repair can be completed, or can be instructed to undergo programmed cell death if DNA damage is too extensive.

In its simplest sense, then, the cell cycle appears to be regulated by a cell growth and a cell death pathway. In this context, we review two fundamental pathways, the 'pRB pathway' and the 'p53 pathway', respectively, which govern these two processes. As is discussed in greater detail in other reviews in this issue, the importance of these pathways in cellular growth control is underscored by the observation that members of these pathways are found mutated in all human cancers. As will also become evident in the discussion below, while these pathways are typically studied and discussed independently, recent data have revealed an intimate molecular and genetic interaction between these pathways.

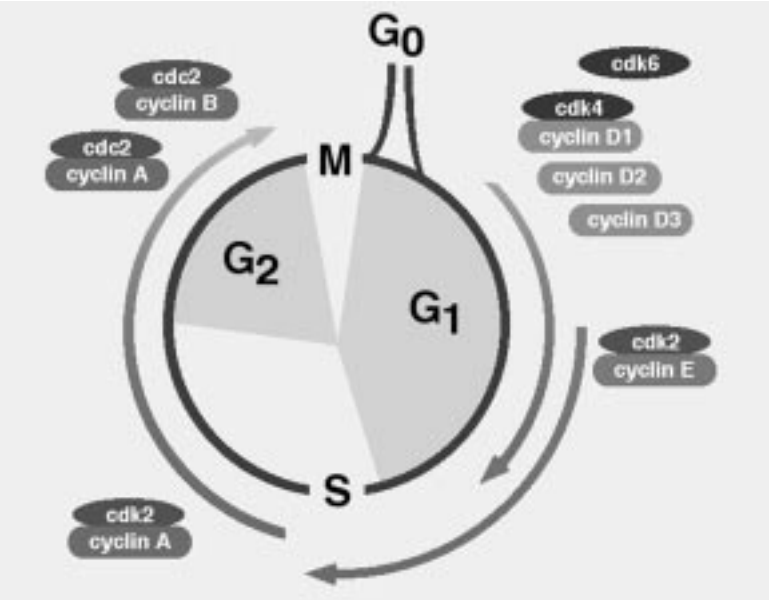

Figure 1. Model for eukaryotic cell division cycle and cyclin expression. A general model depicting the cell cycle used by many, but not all, cells. It is marked by two primary events, DNA synthesis (S phase) and mitosis (M). These phases are separated by gaps, G_1 and G_2. Cell may also leave the cell cycle (G_0) in the absence of mitogenic stimuli to enter a quiescent state or when terminally differentiated. Rate limiting to cell cycle progression is the orderly appearance of the cyclins. The D-type cyclins (cyclins D1, D2 and D3), which associate primarily with the cyclin dependent kinases, cdk4 and cdk6, are expressed first. These are followed following by cyclin E, which binds cdk2 exclusively, at the G1-S boundary. Subsequently, cyclins A and B increase in levels as cells approach M.

The 'pRB pathway'

As has been reviewed in detail elsewhere, rate limiting for progression from G_0/G_1 to S phase is the appearance of the class of proteins, known as cyclins ([3,4]; see [5] for review). For the cell cycle described in Figure 1, these include the D-type cyclins (cyclins D1, D2, and D3) and cyclin E. The appearance of these cyclins following a mitogenic stimulus generally occurs in a highly regulated manner. Failure to express these cyclins results in arrest of the cell cycle at specific points in the cell cycle [6–10]. The early portion of G_1 appears to be governed by the expression of the D-type cyclins [7,11–13]. In fibroblasts, their levels tend to increase significantly, peaking 6–8 h following the mitogenic stimulus. As cells pass through the point governed by cyclin D expression, a second checkpoint at the G_1-S boundary is encountered, this one determined by the expression of a distinct cyclin, cyclin E [9,10,14].

Cyclins D and E are co-factors for a class of kinases known as the cyclin dependent kinases (CDK's). These serine/threonine-specific kinases phosphorylate these residues in the general context of the amino acid sequence Ser/Thr-Pro-x-Arg/Lys [15,16]. Importantly, the cyclins exhibit distinct affinities towards specific members of the CDK family. The D-type cyclins are typically associated with cdk4 [17,18] and cdk6 [17,19] while cyclin E binds cdk2 exclusively [20–22]. Since the peak of associated kinase activity of the D-type cyclins coincides with the restriction point, their expression has been considered to be an essential aspect of the mechanism regulating passage through this point (for example see [23]). This notion is somewhat complicated by the observation that the different D-type cyclins are expressed in distinct but overlapping sets of cell types during embryogenesis and in adult tissues [24–29]. These unique expression patterns account for the defects which are observed in animals deficient for some of these, specifically cyclins D1 [30] and D2 [31]. That these expression patterns reflect unique biological activities is supported by the observations that different sets of D-type cyclins can block cell cycle exit during cellular differentiation in distinct cells [32,33]. It is also clear that the D-type cyclins have other cellular roles distinct from cell cycle regulation, suggested, for example, by the requirement of cyclin D3 for exit of the cell cycle during myogenesis [24,28,34,35].

The pRB family proteins

Returning to mechanisms controlling cell cycle progression, cloning and sequencing of the gene responsible for the pediatric childhood retinal malignancy, retinoblastoma [36], revealed the most important target for cyclin D/cdk4 activity. The human retinoblastoma protein (pRB), harbours 16 distinct cyclin/cdk-consensus sequences. Furthermore, the pRB protein becomes highly phosphorylated at the same point in G_1 that kinase activity associated with the D-type cyclins begins to peak [37–40]. Experiments using both *in vitro* and *in vivo* systems strongly support the notion that the principle target of cyclin D/cdk4 activity is the pRB protein [41–46] and that phosphorylation-dependent inactivation of pRB is required for cell cycle progression [45,47–49]. Whether pRB is important for regulation of cell cycle progression or is more fundamental to cell cycle exit, when, for example, cells terminally differentiate, is unresolved. The latter role is supported by observations where, for many cell types both *in vivo* and *in vitro*, loss of pRB leads to apoptosis during differentiation [50–53]. Compensation for the lack of pRB by the related pRB-family members, p107 and p130, has been proposed for pRB-deficient cells which escape this fate [50,53–56]. Supporting this model are studies employing chimeric mice deficient for pRB and/or p107 [57–59]. Normal retinal development is seen in animals lacking either pRB or p107. In contrast, chimeric animals where both p107 and pRB were absent exhibited hyperplasia, disorganized growth and tumours in the retina, consistent with a compensatory role for p107. However, while p107 and p130 exhibit activities similar to pRB [60–67], their role as tumour suppressors is highly questionable since tumours harbouring mutations in p107 or p130 are, at best, extremely rare [68–70]. Furthermore, mice lacking one or both functional alleles of p107 or p130 are not prone to tumour formation [58,59,71,72].

The E2F-family proteins

Many pRB-interacting factors have been reported (for examples see [73–88]). In the context of the cell cycle, the E2F-family of bHLH transcription factors are the most important if not the best characterized. The six members of this family, E2F-1 to -6, form heterodimers with the DP-family proteins, DP-1, -2 or -3 (for reviews see [89–91]). Their DNA-binding site consensus sequence, first defined in the genome of adenovirus [92], occurs in the promoter region of a large number of factors involved in cell cycle progression or DNA synthesis. Some of these factors include cyclin E [93], dihydrofolate reductase (DHFR; [94–96]), thymidylate synthetase [97], cdc25A [98], cdc2 [99], cyclin A [97], E2F1 itself [100,101], as well as pRB [102–104] and p107 [105]. With the exception of E2F6, all of the E2F-family members have a transcriptional activation domain at their C-termini. The transactivation domain harbours sequences responsible for mediating binding to the pRB-family proteins. Rather than merely repressing the activity of the E2F's, binding of pRB to E2F converts E2F from being an active transcriptional activator to a transcriptional repressor [106,107]. This repressor activity is further enhanced by the association of histone deacetylase [108,109] (HDAC). Thus, active repression also occurs by inducing a 'closed' structure for chromatin at a particular locus. Like the pRB-family and D-type cyclins, the E2F-family proteins also exhibit tissue-specific expression, at least during embryogenesis [110,111]. Animals deficient for specific E2F members have indicated further that E2F3, rather than E2F1, is the principle family member required for cell proliferation during embryogenesis [112–114]. This observation is consistent with antibody microinjection experiments demonstrating that E2F1 is involved in regulation of the first cell cycle immediately following a mitogenic stimulus while E2F3 is required for subsequent cell cycles as cells continue to proliferate [115,116]. It has also been recently shown that in quiescent cells, that pRB is associated primarily with a novel form of E2F3, E2F3b [117], supporting the primacy of this E2F-family member in pRB-dependent cell cycle control. It is clear, however, that E2F1 activity must be carefully regulated. Overexpression of E2F1 or loss of pRB leading to uncontrolled E2F1 activity strongly induces programmed cell death [118–121]. E2F3, in contrast, does not appear to drive apoptosis when its activity is deregulated [116,120,122]. These data suggest further that the different E2F's may have distinct transcriptional targets. This notion is supported by analyses demonstrating distinct perturbations in the expression pattern of different cell cycle regulatory factors using mouse embryo fibroblasts (MEFs) derived from either pRB, p107 or p130-deficient embryos [123].

Thus, while a great number of important details remain to be defined, a fundamental cell cycle regulatory pathway involving the D-type cyclins, pRB-family and the E2F-family proteins has emerged

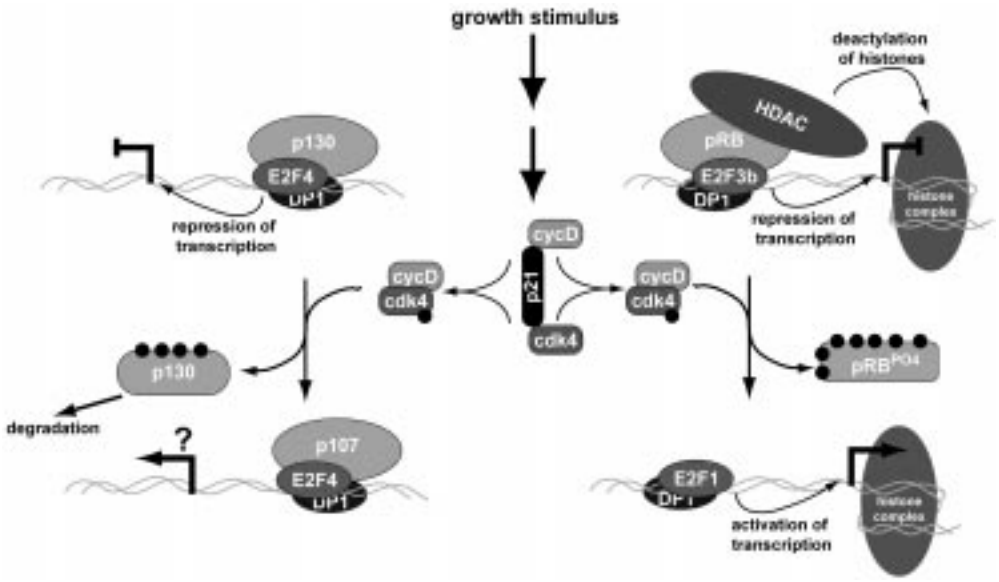

Figure 2. Simple model for the 'pRB pathway'. E2F-family proteins form heterodimers with the DP proteins and, in quiescent cells (G_0), are also associated with pRB-family proteins. Complexes containing E2F4 and p130 predominate although significant levels of pRB bound to E2F3 are also present. Repression is activity of the pRB-containing complexes is further enhanced by co-binding of histone deactylase (HDAC). Cell stimulated to proliferate express the D-type cyclins which associate with the kinase, cdk4 (or cdk6), under the influence of the cyclin dependent kinase inhibitor, p21. The cyclin D/cdk4 co-complex targets the pRB-family proteins for inactivation by mediating their phosphorylation. This inactivation liberates the E2F complexes from the inhibitor activity of the pRB-family proteins. Transcription of factors required for progression through late G_1 into S phase ensues. In the case of E2F4, p130 appears to be replaced by p107 in these complexes. The role of p107/E2F4 complexes in transcriptional regulation of cell cycle factors is not clear.

(see Figure 2). In resting or quiescent cells, complexes of E2F4 associated with p130 appears to be the predominant complex bound to promoters with E2F-consensus sequences [124–126]. pRB-E2F1 co-complexes are also found in quiescent cells, but, given the presence of E2F-binding sites in their promoter regions, their levels tend to be decreased in resting cells via an autoregulatory mechanism . It has also been revealed that pRB or p130-containing complexes exhibit cytoplasmic compartmentalization in specific quiescent cells and terminally differentiated cells both *in vitro* and *in vivo* [127–129]. This compartmentalization further prevents activated transcription by the E2F-family proteins. Transcriptional repression of E2F target genes by pRB/E2F complexes is further enhanced by pRB-dependent binding of histone deacetylase (HDAC).

As cells are stimulated to enter the cell cycle, the p107/E2F4 complexes replace the p130/E2F4 [130] while complexes containing pRB and E2F1, E2F2 or E2F3, bound to DNA, become more significant [126]. The inhibitory effects of the pRB-family proteins on E2F-dependent activated transcription are then lost due to the appearance of cyclin D. In combination with cdk4/6, phosphorylation of the pRB-family proteins liberates E2F from these repressors, resulting in the activated transcription of E2F target genes. It has been suggested further that another checkpoint involves E2F1 (and presumably E2F2 and E2F3) during S phase [131,132]. Specifically, following entry into S, E2F activity appears to be inhibited by binding to E2F1 of cyclin A, whose levels begin to increase during S. The cyclin A/cdk2 complex phosphorylates the DP proteins and possibly E2F, decreasing E2F/DP affinity for DNA and, thereby, causing its release. This model for regulation of E2F1 activity during S was supported using a mutant E2F1, deficient for the cyclin A binding site. This mutant promoted cell cycle progression in resting cells but blocks their exit from S phase.

Thus, while specific temporal aspects of this pathway are under active investigation, it is clear that the 'pRB pathway' represents an important growth control mechanism involving the antagonistic cell cycle

regulatory activities of the D-type cyclins, the pRB-family proteins and the E2F-family of transcription factors.

The 'p53 pathway'

The p53 protein

The transcription factor, p53, acts as a fundamental regulator of cell cycle arrest and apoptosis in the normal cell. It's central role in these processes is supported by the fact that p53 is the most frequent target for inactivation in malignantly transformed cells. Since its discovery in 1979, p53 mutations have been described in more than 50% of human cancers [133]. While its complete role continues to be elucidated, it is clear that p53 integrates signals from internal and external stimuli, allowing the cell to respond to a variety of stresses. These responses are generated, in part, by p53-mediated transcriptional activation of genes possessing a p53-response element in their promoter.

Structurally, the human p53 transcription factor is 393 amino acids long and consists of five domains (for review, see [134]). The first 42 amino acids at the N-terminus constitute the transactivation domain which interacts and co-operates with components of the basal transcription machinery such as TAFs (TATA-binding protein Associated Factor). Furthermore, inhibition of p53-mediated transcription is achieved through binding of proteins to the p53 transactivation domain. These negative regulators of p53 activity inhibit transcription by both interaction with the p53 transactivation domain and direct inhibition of the transcriptional machinery assembled at the promoter.

In conjunction a proline-rich domain (PRD) located between the transactivation domain and the sequence-specific DNA-binding domain, the C-terminal domain (CTD) regulates the growth arrest and apoptotic promoting activities of p53. The CTD harbours basic residues that bind preferentially to specific DNA and RNA sequences and to DNA ends. In addition, this domain mediates the reassociation of double-stranded DNA or RNA from single strands. The CTD in conjunction with the PRD maintains the p53 tetramer in a conformation that has low-affinity for binding its consensus sequence (5′-PuPuPuC(A/T)-3′ arranged as a pair of inverted repeats). Phosphorylation by protein kinase C or casein kinase II activates sequence-specific DNA binding of p53 resulting in activated transcription of, for example, $p21^{Cip1/WAF1/Sdi1/Cdi1}$ (p21), MDM2, GADD45, Cyclin G, Bax, and IGF-BP3 [135–140]. In addition to its transactivation activity, p53 can also repress the expression of several cellular and viral genes whose promoters do not contain a p53-response element [141]. Among these genes are c-fos and SV40 large T antigen [142–144].

The p53 inhibitor, MDM2

Under normal conditions, p53 is a latent, short-lived protein with a half-life of 5–20 min. Protein levels and activity are kept low through various regulatory mechanisms. One of the primary regulators of p53 function in the cell is the Murine Double Minute-2 protein, or MDM2. MDM2 was originally discovered as a gene overexpressed in the tumourigenic 3T3DM mouse cell line that stably maintains double minute chromosomes [145]. The N-terminus of MDM2 binds to the transactivation domain of p53 [146,147]. This interaction inhibits the transcriptional activity of p53 through masking of the transactivation domain, and direct inhibition of the basal transcription machinery at the promoter, possibly TFIIE [148–150]. The transrepression function of p53 is also impaired as a consequence of MDM2 binding [151]. The critical role of MDM2 in the modulation of p53 activity is most evident in the fact that the embryonic lethal phenotype seen in MDM2-null mice can be overcome by co-deletion of p53 [152].

In addition to modulating p53 activity, MDM2 regulates p53 protein levels. Binding of MDM2 to p53 targets p53 for nuclear export and subsequent degradation in the cytoplasm [153–155]. MDM2 contains a nuclear export signal that allows it to co-transport p53 out of the nucleus into the cytoplasm [156–158]. There, MDM2 functions as an E3 ubiquitin ligase, directly targeting p53 for destruction via the ubiquitin-proteosome degradation pathway [159–162]. However, as noted above, MDM2 is a transcriptional target of p53. Thus, an autoregulatory loop exists where high p53-mediated transactivation is countered by p53-dependent up-regulation of the p53 inhibitor, MDM2 [163] (see Figure 3).

The cellular response to DNA damage illustrates the intimate relationship between p53 and its regulator MDM2 in the presence of stressful stimuli. Genetic insults derived from ionizing radiation compromise the integrity of genomic DNA structure, catalyzing breakage of DNA double-strands. The free DNA ends created as a result leads to series of events, one of which is

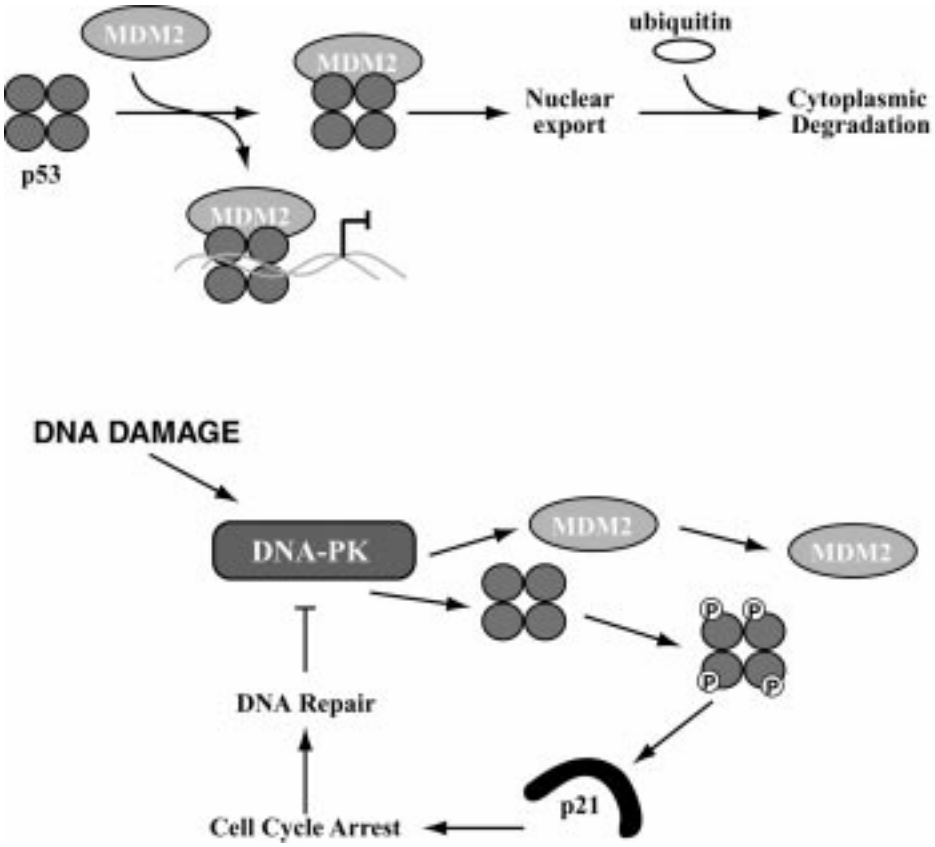

Figure 3. Simple model for the 'p53 Pathway'. Upper panel: Under normal conditions, MDM2 binds to the transactivation domain of p53 and inhibits p53-mediated transactivation. MDM2 also functions to keep levels of p53 protein low by targeting p53 for ubiquitin-dependent proteasomal degradation in the cytoplasm. Lower panel: Genetic insults which damage genomic DNA structure activate enzymes such as DNA-PK. Activated DNA-PK phosphorylates residues in the N-terminal regions of p53 and MDM2, thereby blocking the interaction between these two proteins. Consequently, levels of p53 stabilize and induce transactivation of cell cycle regulatory genes. Induction of $p21^{Cip1}$ arrests the cell cycle until DNA damage is repaired, at which time DNA-PK activity levels decrease due to the absence of compromised DNA structure. As a result, newly-synthesized p53 and MDM2 remain unphosphorylated and p53 regulation reverts back to that depicted in the upper panel as the cell cycle progresses.

activation of a nuclear kinase known as DNA-PK. Both p53 and MDM2 undergo phosphorylation mediated by DNA-PK (or enzymes with similar specificity) at their respective N-terminal regions after DNA damage [164,165]. As a result, p53 and MDM2 fail to bind each other, leading to stabilization of p53 protein in the nucleus and thereby causing activated transcription of target genes that induce either cell cycle arrest [137] or apoptosis [166–168]. When p53 elicits cell cycle arrest through activation of genes such as p21 (see below) the proliferative block is overcome only when damaged DNA is repaired. Once repaired, DNA-PK activity decreases due to the loss of DNA ends [164]. Consequently, newly-synthesized p53 and MDM2 would remain unphosphorylated leading to decreased p53 stability, protein levels and overall activity as the cell cycle progresses once again.

The Cip/Kip family of CDK inhibitors

A critical mediator of the p53 response to DNA damage is the CDK inhibitor p21 (cloned variously as Cip1 [169], Cdi1 [170], Sdi1 [171] and WAF1 [172]). MEFs derived from p21-null animals fail to undergo normal G_1 arrest in response to DNA damage [173]. Moreover, p21 has been implicated in protecting cells from apoptosis initiated from stress or p53 induction, although the mechanisms remain obscure [174,175]. p53 directly transactivates expression of p21 via p53 binding sites

in the p21 promoter [172,176]. That p21 is transcriptionally regulated by p53 provided an important link between the function of the major human tumor suppressor and negative cell cycle control. However, a basal level of p21 can be found in cell derived from p53-deficient mice indicating that p21 expression is also regulated in a p53-independent manner [177–179]. One of these p53-independent pathways involves TGF-β1. Here, cell cycle arrest is mediated, at least in part, by the direct induction of p21 via TGF-β1 binding sites in the promoter region of p21 [180].

p21 can function as a dual specific inhibitor of cell proliferation by two independent and functionally distinct mechanisms. In addition to its ability to bind and inhibit CDK's, p21 also associates with the DNA replication factor PCNA via the unique carboxyl-terminal domain in PCNA [181]. PCNA is an auxiliary protein to DNA polymerase-δ required for DNA synthesis [182]. Overexpression of the C-terminal domain of p21 in mammalian cells reduces the fraction of cells found in S phase [183]. Furthermore, *in vitro*, the p21/PCNA interaction blocks DNA replication catalyzed by the pol-δ/RFC/PCNA complex [181] but does not inhibit PCNA-mediated DNA repair [184]. There are six binding sites for p21 per PCNA trimer [185], therefore p21 can form either a quaternary complex with PCNA, cyclin and CDK or can bind to PCNA directly. The fact that complexes containing p21 and cyclin/CDK's also include PCNA suggests that p21 may coordinate CDK-dependent cell cycle progression with processes regulating DNA replication and/or repair.

p21 is one member of the Cip/Kip family of CDK inhibitors, a family that includes $p27^{Kip1}$ (p27) and $p57^{Kip2}$ (p57). In contrast to the INK4 proteins, the Cip/Kip family members inhibit a wide range of cdk's which include cdk4, cdk6 and cdk2 [186]. In addition, the two families also differ in their mechanism of binding to CDK's. Cip/Kip proteins inhibit kinase activity by making contact with both the cyclin and CDK subunit [185–189]. Structurally, all three members of the Cip/Kip family have a 65-amino-acid region with homology (38–44% identity) at their N-terminal portions, which is necessary and sufficient for binding and inhibition of G_1 cyclin/CDK complexes [190] as well as cyclin B-containing complexes [187]. However, unlike the INK4 proteins, which demonstrate extensive sequence similarity and functional redundancy, each Cip/Kip family member has distinct functional properties, attributable to structural differences at their C-termini. While only p21 is strictly in the 'p53 pathway', we briefly describe here the other Cip/Kip family members for completeness.

$p27^{Kip1}$ (p27), like other members of the Cip/Kip family, has a CDK-binding domain at its N-terminus, which binds to and inhibits cyclin D-, E-, A-, and B-dependent kinases [191–194]. This inhibitor shares 47% amino acid identity with p21. In contrast to p21, p27 does not bind PCNA and is not regulated by p53. The expression of p27 is controlled, in part, post-translationally [195,196]. p27 mRNA is also induced by vitamin D3 in U937 cells [197] and by IFNβ and IFNα [198,199] suggesting that transcriptional regulation of the p27 gene is also important during cellular differentiation and inhibition of cell growth. The expression of p27 is high in cells inhibited by cell contact, serum deprivation and by a cAMP activated pathway [200–203].

In proliferating cells, p27 is found predominantly in complexes with cyclin D-cdk4/6 [193,204]. These complexes are active, perhaps as a result of p27 being bound to the cyclin subunit without establishing an inhibitory interaction with the CDK subunit. TGFβ treatment of these cells does not increase the total level of p27, but induces a redistribution of p27 from cyclin D/cdk4/6 complexes to cyclin E-cdk2, thereby inhibiting cdk2 [205,206]. As will be discussed in more detail below, this redistribution occurs as a result of a rapid induction of $p15^{INK4b}$ by TGFβ1 [206]. In contrast to its effect on cyclin D/cdk4/6 complexes, p27 has a potent inhibitory influence on cdk2-containing complexes. The crystal structure of p27 bound to the cyclin A-cdk2 complex revealed that p27 invades the catalytic subunit and dismantles its ATP binding site [190]. Hence, cyclin D-cdk4/6 can sequester p27 without being subjected to inhibition, whereas the catalytic activities of complexes containing cdk2 are efficiently abolished by the same CDK inhibitor. Since cell cycle progression requires cyclin E- and A-associated kinase activity, reduction of p27 levels is required. Loss of p27 occurs analogously to other cell cycle regulatory factors, specifically via a ubiquitin-mediated pathway in late G_1 [207–209].

It is likely that the weak inhibition of cyclin D/cdk4 by p21 or p27 is due to the role of these CKI's in formation of these cyclin D/cdk4/6 complexes [210]. As Figure 2 depicts, p21 and p27 promote interactions between the D-type cyclins and their CDK partners by stabilizing the complexes and acting as chaperones for their transport to the nucleus [211,212]. Assembly of cyclin D1/D2-cdk2 complexes is impaired in primary MEFs taken from animals lacking p21, p27 or

both [210]. Moreover, lack of cdk4 in cdk4-deficient mice coincides with increased binding of p27 to cyclin E/CDK2, diminished activation of cdk2 and impaired pRB phosphorylation [213]. These data suggest that one rate-limiting cdk4-dependent mechanism controlling the the G_0 to S transition involves regulation of p27 activity. Thus, the Cip/Kip proteins act as positive cell cycle regulators, facillitating cyclin D-cdk complex formation. They are also potent inhibitors of cell cycle progression when they block kinase activity associated with cyclin E/cdk2 or cyclin A/cdk2 complexes.

The most recently identified member of the Cip/Kip family is $p57^{Kip2}$ (p57; [214,215]). It harbours an N-terminal cdk inhibitory domain and has sequences similar to p27 at its C-terminus. Like p21, p57 contains a PCNA-binding domain within its C-terminus that, when separated from its N-terminal CDK-cyclin binding domain, can prevent DNA replication *in vitro* and S phase entry *in vivo* [216]. Disruption of either cdk/cyclin or PCNA binding partially reduces the ability of p57 to suppress myc/RAS-mediated transformation in primary cells, while loss of both inhibitory functions completely eliminates it's suppressive activity. p57 is a potent inhibitor of the G_1- and S-phase cdk's (cyclin E/cdk2, cyclin D2/cdk4, and yclin A/cdk2) and, to lesser extent, of the mitotic cyclin B/cdc2 [214,215]. The ability of p57 to inhibit cyclin D/cdk4 complexes as well as cdk2-containing complexes appears to be due to the utilization in p57 of a 3(10) helix region for its inhibitory activity [217]. Mutations within the 3(10) helix region of the p57 molecule completely abolish its ability to arrest the cell cycle at G_1 *in vivo*, whereas deletion of the analogous structure in either p21 or p27 has no effect on their ability to inhibit cdk2-associated kinase activity.

Thus, the 'p53 pathway' appears to be a fundamental pathway which regulates cell cycle progression in response to cellular (DNA) damage. While it appears to have a limited role in the normal control of progression, it is clearly fundamental for maintaining the integrity of the genome and, in the event of a catastophic insult, essential for driving cells into the programmed cell death pathway.

The cell growth and cell death pathways are controlled by a single genetic locus

As described in the introduction above, the 'pRB pathway' and the 'p53 pathway' have typically been described and studied independently. However, the discovery that a single genetic locus, the *CDKN2A* locus, which produces two unrelated proteins, one of which regulates the pRB pathway and the other the p53 pathway, provided the first evident of the interrelationship between these cell growth and cell death pathways (see Figure 4). We now discuss the INK4 family of cdk4-specific inhibitors and the ARF protein which is responsible for regulating mdm2 activity.

The INK4 family proteins

The INK4 family of CDK inhibitors are 15- to 19-kDa proteins which specifically inhibit cdk4 and cdk6 kinase activity (hence the nomenclature INK4 = INhibitor of Cyclin Dependent Kinase 4). The prototype inhibitor, $p16^{INK4a}$, was isolated in a yeast two hybrid screen of a HeLa cell cDNA library with cdk4 [218] and as the candidate gene mutated in familial melanoma [219]. The INK4 family currently includes four members: $p15^{INK4b}$ [220,221], $p16^{INK4a}$, $p18^{INK4c}$ [222], and $p19^{INK4d}$ [222,223]. Structurally, the INK4 inhibitors are closely related, sharing 40% amino acid identity between them. The important functional motif common to these proteins is their ankyrin-like repeats which mediate protein–protein interactions specifically with cdk4 or cdk6. $p15^{INK4b}$ and $p16^{INK4a}$ have four of these repeats while $p18^{INK4c}$ and $p19^{INK4d}$ have five repeats. Inhibition of kinase activity by the INK4 family members is mediated by direct binding of the inhibitor, particularly via the third ankyrin-like repeat, to cdk4 or cdk6. The solved crystallographic structure of the $p19^{INK4d}$: cdk6 [224] and $p16^{INK4a}$: cdk6 [225] binary complexes revealed that the INK4 proteins bind to the side opposite the cyclin binding face of the CDK. Binding induces significant distortion between the N- and C-terminal lobes of the CDK and further prevents the 'PSTAIRE' α-helix from participating in formation of the catalytic cleft. These distortions prevent binding of cdk4 or cdk6 to the cyclin and block any possibility of the kinase having catalytic activity. Blocking CDK association with the cyclin was, in fact, predicted based on biochemical analyses prior to generation of the crystallographic data [226–228].

In vivo, the INK4 proteins are found in complexes containing cdk4 or cdk6 unbound by cyclin D. The INK4 proteins are also capable of inhibiting pre-assembled cyclin D/cdk4/6 complexes as well [206,229]. *In vitro*, these trimeric structures were devoid of kinase activity, consistent with significant

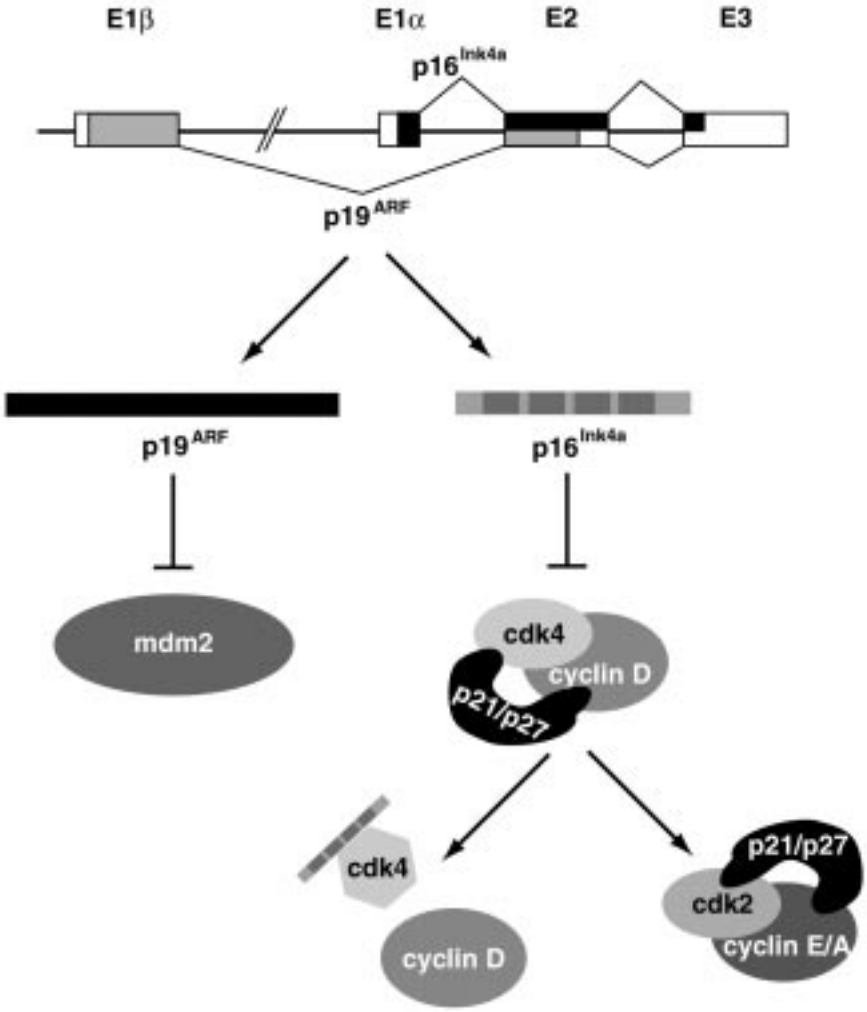

Figure 4. p19ARF and $p16^{INK4a}$ are Expressed From the Same Genetic Locus. Through alternative splicing, the CDKN2 locus produces transcripts encoding the cdk4 inhibitor, $p16^{INK4a}$, and the mdm2 inhibitor, $p19^{ARF}$. Binding of the cdk4-specific Ink4 inhibitors induces significant structural alteration in cdk4 such that it no longer binds to the cyclin. One consequence of this dissociation is the mobilization of p21 or p27, which chaperoned formation of the cyclin D/cdk4 co-complex. p21 and p27 can are then able to bind to and inhibit complexes containing cyclins E or E and cdk2.

distortion of the catalytic cleft of the kinase due to INK4 binding [224,225].

Analogous to other cell cycle regulatory protein families, the INK4 proteins have distinct expression patterns in developing mice despite their similarities in structure and function. Transcripts encoding $p15^{INK4b}$ and $p16^{INK4a}$ are not detected during embryogenesis, but low levels of $p15^{INK4b}$ and $p16^{INK4a}$ mRNA are discretely expressed in adult lung, testis, spleen and kidney [230,231]. Furthermore, expression of $p16^{INK4a}$ transcript and protein increases as mice grow older, implicating a role for this particular INK4 protein in cellular senescence. In contrast to $p15^{INK4b}$ and $p16^{INK4a}$, transcripts encoding $p18^{INK4c}$ and $p19^{INKd}$ are detectable during embryonic development and in a wide variety of postnatal tissues which include the heart, testis, spleen, lung and skeletal muscle [230]. Interestingly, $p18^{INK4c}$ expression in murine brain is restricted to dividing neurons, while $p19^{INK4d}$ is present primarily in post-mitotic neurons [231]. Amongst the INK4 proteins, $p19^{INK4d}$ expression levels predominate in the adult brain.

In the context of normal embryonic development, only $p19^{INK4d}$-null mice show a significant phenotype, specifically exhibiting testicular atrophy [232]. Other INK4-knockout mice have revealed that, biologically, the INK4 proteins are not completely redundant, but rather may have lineage-specific functions *in vivo* [232–234]. Detailed discussion of the consequences of INK4-family knock-out animals specifically on tumour growth can be found in the review by Ivanchuck and Mondal.

While the cell cycle arrest following expression of $p16^{INK4a}$ occurs specifically through inactivation of the cyclin D/cdk4/6 complexes, binding of the INK4 proteins to cdk4 and cdk6 indirectly leads to inhibition of cyclin E/cdk2 and cyclin A/cdk2 complexes. This activity of the INK4 proteins is apparently mediated by mobilization of the Cip/Kip family of CDK inhibitors (see Figure 4; [235]). Specifically, as described above, the Cip/Kip proteins chaperone the formation of cyclin D/cdk4/6 complexes, apparently associating with active cyclin D/cdk4/6 complexes in a 1:1 ratio without impairment of kinase activity [212,236]. However, induction the INK4 inhibitors competes with Cip/Kip proteins for binding to cdk4/6. The INK4 proteins bind cdk4/6 in the cytoplasm, blocking subsequent Cip/Kip association to these kinases. The inability to bind cdk4/6 then mobilizes the once latent pool of Cip/Kip inhibitors, re-distributing them to cyclin E/cdk2 and cyclin A/cdk2 complexes [235]. These latter cyclin/cdk complexes are more sensitive to inhibition by Cip/Kip proteins than the cyclin D/cdk4/6. Thus, expression of the INK4 proteins leads to concomitant loss of virtually all G_1 cdk activity and effectively inducing cell cycle arrest.

These findings bring into question the relative importance of cdk4 versus cdk2 kinase activity during proliferation. Does direct inhibition of cdk4/6 by the INK4 proteins, or the subsequent loss of cdk2 activity mediated by Cip/Kip proteins elicit cell cycle arrest? A number of data support the latter possibility. For example, a catalytically inactive version of cdk2 acts in a dominant manner, blocking proliferation [237] while the analogous cdk4 mutant has no effect on the cell cycle progression [18]. Furthermore, ectopic expression of $p16^{INK4a}$ fails to arrest cells programmed to overexpress cyclin E [238]. Finally, $p16^{INK4a}$ expression in the U2-OS osteosarcoma cell line induces cell cycle arrest that is associated with a corresponding induction of p21 protein levels and subsequent inhibition of cdk2 activity [239]. These results begin to suggest that the Cip/Kip proteins play a more prominent role as negative cell cycle regulators.

The ARF tumour suppressor

Genetically, the $p16^{INK4a}$ protein encoded by the *CDKN2A* locus exerts its influence at the 'top' of the pRB pathway. Northern analysis revealed, however, that a related transcript was also produced from this locus. This message encoded a second unrelated protein, $p14^{ARF}$ [240]. The relationship between $p16^{INK4a}$ and $p14^{ARF}$ is depicted in Figure 4. $p16^{INK4a}$-transcripts are generated by splicing of exon E1α to exons 2 and 3 of the locus. Through the use of a distinct first exon, exon E1β, located 20 kilobase pairs upstream of exon E1α, *CDKN2A* also encodes for a 14 kDa cell cycle inhibitor, the expression of which is regulated by a separate promoter [241] and translated in an alternate reading frame of exon 2 [242]. In humans, this protein is known as the p14-Alternate Reading Frame product, or $p14^{ARF}$, while the larger murine homologue is referred to as $p19^{ARF}$ [242,243]. For simplicity, we will refer to the murine and human proteins collectively as ARF unless they need be distinguished. It should be noted that recent studies have shown that the human *CDKN2A* locus encodes a third transcript using exon E1α and 274 base pairs of intron 1 which generates a 12 kDa protein expressed specifically in the pancreas referred to as p12 [244]. It has been suggested that p12-dependent cell cycle arrest may be independent of the pRB and p53 pathways, based on its effect when expressed in the pRB- and p53-deficient, human cervical carcinoma, C33A.

Structurally, ARF is a highly basic protein that shows no structural similarities to known proteins in searchable databases. To date, all known growth suppressive functions of ARF are encoded by exon 1β. *In vivo*, ARF is a nuclear protein localized specifically to the nucleolus [240]. A consensus nucleolar localization signal is present in exon 1β of $p19^{ARF}$ [245] while this signal is found in exon 2 of $p14^{ARF}$ [246]. ARF cell cycle inhibitory activity is mediated through the 'p53 pathway' by indirectly stabilizing and activating p53 [243]. Specifically, ARF binds and sequesters MDM2 in the nucleolus, preventing MDM2-mediated export of p53 to the cytoplasm for degradation [247–252]. This sequestration of MDM2 may be promoted in part by a nucleolar localization signal within the MDM2 C-terminal RING domain, which is unmasked upon ARF binding [245]. The interaction of ARF with MDM2 also inhibits the ubiquitin ligase activity of

MDM2, allowing p53 to escape ubiquitin-mediated proteosomal degradation [248]. In addition to stabilizing p53 protein levels, ARF activates p53-dependent transcription by impairing the ability of MDM2 to inhibit p53 transactivation of targets [247,252]. Consequently, expression of p53 target genes, such as p21, is up-regulated, inducing cell cycle arrest in both G_1 and G_2/M. Furthermore, ectopic overexpression of $p19^{ARF}$ in cells containing wild-type p53 blocks cell cycle progression in G_1 and at the G_2/M boundary [242,253]. This ARF-mediated p53 activation can be regulated through modulation of the activity of the ARF promoter. Wild-type p53 can down-regulate transcription from the $p14^{ARF}$ promoter despite the fact that this promoter does not appear to have p53 binding sites [241]. Thus, an autoregulatory feedback loop is formed in which $p14^{ARF}$ activates p53, the latter of which can then down-regulate p14ARF transcription to ensure that p53 levels remain in check [241,243]. Finally, the Bmi-1 Polycomb-group transcriptional repressor also functions as a negative regulator of ARF (and $p16^{INK4a}$) expression. Overexpression of Bmi-1 down-regulates $p19^{ARF}$ expression, while levels of $p19^{ARF}$ protein increase in the absence of Bmi-1 [254,255].

The participation of ARF in specific signaling pathways upstream of p53 requires further elucidation. While it is clear that ARF expression is induced in response to hyperproliferative signals, the role of ARF in the p53-mediated cellular response to DNA damage is being challenged. DNA damage induced in mouse embryo fibroblasts (MEFs) derived from a $p19^{ARF}$-specific nullizygous mouse ($p16^{INK4a}$ expression is intact in this animal) causes p53 activation, p21 accumulation and subsequent cell cycle arrest in a manner identical to that of their wild-type counterparts [242]. More recent experiments examining the DNA damage response of $p19^{ARF}$-null MEFs over a substantially longer time course have demonstrated that these cells continue cycling 24 h post-exposure to ionizing radiation relative to untreated control cells [256]. The sustained induction of p53 observed up to 48 h after radiation exposure of wild-type MEFs was not observed in $p19^{ARF}$-null MEFs. Instead, induction of p53 protein expression in $p19^{ARF}$-null MEFs transiently increased 2–10 h post-irradiation, but decreased to undetectable levels after 24 h. This correlates with the finding that levels of p21 protein increased 2- to 5-fold in wild-type MEFs, while the maximal increase seen in $p19^{ARF}$-null MEFs was only 2-fold. The participation of $p19^{ARF}$ in the p53-mediated response to DNA damage is further substantiated by the induction of $p19^{ARF}$ protein levels in wild-type MEFs 2–10 h following exposure to ionizing radiation. Thus, the role of ARF as an upstream activator of p53 in cell cycle regulation may include the cellular response to DNA damage.

The absence of ARF expression during murine embryogenesis suggests that ARF does not play a role in development. Instead, ARF mRNA, like that of $p16^{INK4a}$, is detected postnatally in limited tissues such as the testis and lung [230]. As mice grow older, levels of $p19^{ARF}$ transcript increase in the brain, but are unchanged in most other tissues. This would suggest that ARF might participate primarily in maintaining the arrested state of specific cell lineages *in vivo*. In accordance with this, induction of ARF expression, p53 stabilization and subsequent growth arrest occurs in wild-type MEFs, but not p53-null MEFs, in response to oncogenic signals such as E1A, Myc, v-Abl, and Ras [257–260]. Following Myc transformation, ARF enhances the apoptotic response of Myc-expressing wild-type MEFs following withdrawal of serum. In addition, it appears that ARF can also co-operate with other growth inhibitors to combat tumourigenesis as is evident in the ability of the BRCA1 breast tumour suppressor to induce $p14^{ARF}$ expression in the H460 human lung non-small cell carcinoma cell line [261].

Interactions between the pRB and p53 cell cycle control pathways

ARF regulates the cell cycle through both the pRB and p53 pathways

To date, the ability of ARF to arrest the cell cycle through p53-dependent mechanisms has been well characterized. ARF antagonizes the negative regulatory function of MDM2 to stabilize and activate p53, and thereby inhibits proliferation through the p53 pathway (see Figure 5). MDM2, however, in addition to its interaction with p53, binds to the C-terminus of pRB [262]. Binding of MDM2 to pRB inhibits the regulation of E2F1 activity by pRB, and can overcome a pRB-induced G_1 arrest in U2-OS cells. Furthermore, MDM2 directly stimulates the transcriptional activity of E2F1 through contacts made with the activation domain of E2F1 [263]. *In vivo*, binary complexes containing MDM2 bound to either E2F1 or DP-1 are seen, and the direct interaction of MDM2 with E2F1 is necessary for MDM2-mediated stimulation of E2F1 transcriptional activity. In addition to this, MDM2 may

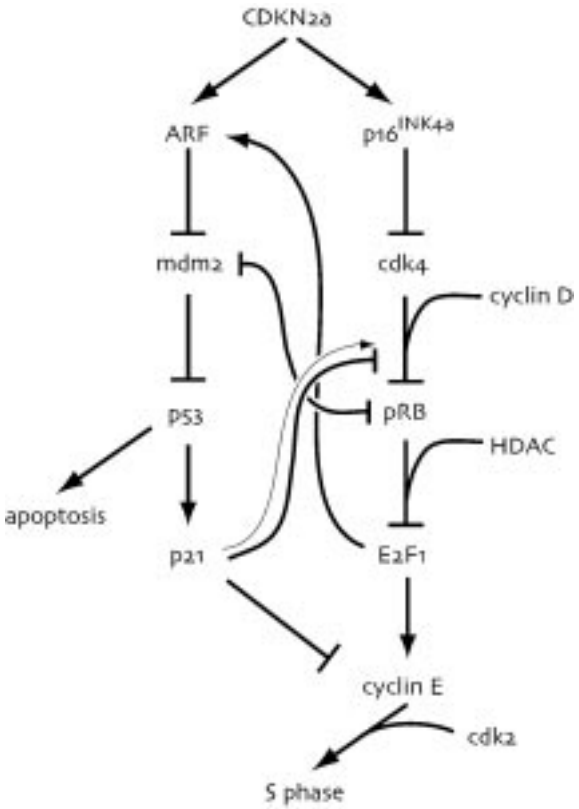

Figure 5. Integrated p53 and pRB pathways. Activities of the factors in the 'pRB pathway' influence the expression and activity of a components of the 'p53 pathway'. So, for example, E2F1 causes increased expression of $p19^{ARF}$ while pRB has a negative influence on the ability of mdm2 to regulate p53 activity. Likewise elements of the 'p53 pathway' alter the activity of the 'pRB pathway'. Mdm2 binds to and regulates pRB activity and the p53-regulated CDK inhibitor, p21, is required for assembly of cyclin D/cdk4 complexes. Recent genetic data have also demonstrated that the important regulator of the p53 pathway, $p19^{ARF}$, requires that the pRB pathway be intact. See the text for details.

indirectly stimulate E2F1 as a consequence of the nature of the MDM2 : pRB interaction. MDM2 binds to the pRB C-terminal domain, which is the region also required, along with the pRB 'small pocket', for complex formation with E2F1 [264–266]. Therefore, interaction of MDM2 with pRB would maintain E2F uncomplexed with pRB, and allowing transcription of factors required for cell cycle progression [249]. The autoregulatory aspect of E2F1 described above might is consistent with the ability of MDM2 to increase E2F1 transcriptional activity. This activity may be further enhanced by the apparent ability of MDM2 to participate in the stabilization of E2F1 protein under certain conditions [267]. TGF-β treatment of Mv1Lu cells induces a decrease in E2F1 activity and protein expression, both of which can be prevented by ectopic expression of MDM2 in these same cells. Therefore MDM2, in addition to being a primary inhibitor of p53, enhances E2F activity through both direct and indirect mechanisms. The ability of MDM2 to act on both the p53 and pRB tumour suppressor proteins makes it functionally analogous to SV40 Large T antigen.

Further evidence supporting the notion that ARF interacts with the pRB pathway continues has been recently published. Induction of $p19^{ARF}$ expression in NIH 3T3 cells correlates with a p53-mediated increase of cdk2-bound p21 and a commensurate increase in hypophosphorylated pRB [252]. That E2F1 can directly induce $p14^{ARF}$ expression through an E2F-binding site in the $p14^{ARF}$ promoter region, suggests that ARF can function downstream of pRB. This notion is further supported by the finding that overexpression of E2F1 in normal human fibroblasts up-regulates $p14^{ARF}$ transcript and protein and induces a senescent phenotype.

Recent studies have revealed that ARF can function in a p53-independent manner to suppress growth through the pRB pathway by virtue of ARF's ability to antagonize MDM2 function [268]. Expression of dominant-negative p53 was incapable of overcoming the proliferative block induced upon restoration of $p19^{ARF}$ function in MEFs. Likewise restored expression of $p19^{ARF}$ in p53-null MEFs induced growth arrest, which could be overcome by simultaneous inactivation of $p16^{INK4a}$ using antisense mRNA or overexpression of E2F1. Furthermore, a fraction of immortal clones derived from p53-null MEFs had lost or downregulated $p19^{ARF}$ mRNA, demonstrating that, in the absence of functional p53, there is still selective pressure to inactivate $p19^{ARF}$ during the process of immortalization. This apparent p53-independent, pRB-dependent mechanism for ARF-mediated growth arrest is contrary to the initial hypotheses surrounding ARF function.

Regulation of the p53 pathway by pRB

The biological consequences of the pRB : MDM2 interaction can be viewed from two perspectives. As discussed previously, binding of MDM2 to the C-terminal domain of pRB inhibits the ability of pRB to negatively regulate E2F activity. However, recent evidence suggests that pRB impairs certain functions of MDM2 during the process of forming a trimeric

complex with p53. Specifically, pRB overcomes the ability of MDM2 to inhibit p53-mediated apoptosis [269]. In the pRB- and p53-deficient human osteosarcoma, Saos-2, the percentage of cells containing a sub-G_1 DNA content (an indicator of apoptosis) was decreased by 50% by the addition of MDM2 to p53-transfected cells. This decrease in apoptotic cells was reversed by co-expression of pRB, suggesting that pRB binding to MDM2 could block MDM2 anti-apoptotic activity. This notion was supported by the ability of pRB expression to maintain p53 stability despite expression of MDM2. These results suggest that pRB inhibits MDM2-mediated p53 degradation. However, pRB does not impair all of the inhibitory effects of MDM2 on p53. With respect to p53 transcriptional activity for example, pRB blocks the ability of MDM2 to impair transcriptional repression mediated by p53 but does not appear to alter MDM2's inhibition of p53-mediated transcriptional activation [269]. The latter observation may be explained by the nature of the trimeric complex which is formed through binding of pRB and p53 to non-overlapping regions of MDM2. Binding of pRB to MDM2 does not promote the dissociation of p53 from the latter [269]. Thus, even in the trimeric complex, MDM2 remains bound to the p53 transactivation domain and thereby continues to inhibit the transcriptional activity of p53 by masking this domain despite the binding of pRB.

That the pRB pathway has a significant interaction with the p53 pathway is further substantiated by the finding that MDM2 binds preferentially to hypo-phosphorylated pRB [269,270]. This implies that events which function to activate pRB, such as expression of $p16^{INK4a}$ or mitogen depletion, cause subsequent activation of p53 through inhibition of MDM2 function in addition to down-regulation of E2F activity. We suggest that the ability of the pRB pathway to regulate the activity of p53 may provide an explanation for the induction of p21 protein levels following expression of $p16^{INK4a}$ in U2-OS cells described previously. Furthermore, the extensive interactions between the two pathways permits each pathway to compensate for defects in the other. In the absence of functional p53, levels of p21 diminish such that the formation of cyclin D/cdk4/6 complexes and their subsequent transport to the nucleus would be perturbed, leading to activation of pRB. Similarly, the loss of pRB function would be compensated for by E2F1-mediated up-regulation of ARF expression which would induce p53 activation. However, the disruption of only one of these pathways can lead to malignant transformation, and thus the degree to which these compensatory measures extend warrants further study and points to the existence of other growth regulatory pathways which are important in the maintenance of cell cycle control.

To conclude, it is clear that two fundamental pathways, the pRB and the p53 pathways, regulate cell growth and cell death. As is evidenced by the continuous publication of important papers in this area, only very broad outlines of the mechanisms controlling these pathways have been defined. It is also clear that both pathways must be simultaneously considered during discussion of cell cycle control given the recent data which clearly demonstrate the molecular and genetic interactions between these two pathways.

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Address for correspondence: Paul A. Hamel, 6318, Medical Sciences Building,1 King's College Circle University of Toronto, Toronto, Ontario, Canada M5S 1A8; E-mail: paul.hamel@ utoronto.ca