FUNCTIONAL CHARACTERIZATION OF THE CYCLIN-DEPENDENT KINASE INHIBITOR p21 BY GENE TARGETING IN THE MOUSE

by

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ABSTRACT

The cyclin-dependent kinase inhibitor p21 is a negative regulator of cell proliferation that has been implicated in tumor suppression, differentiation and senescence. To understand the role of p21, we generated a mouse strain lacking the coding sequence of the *p21* gene and characterized both this strain as well as fibroblasts derived from these mice. Whereas *p21*-deficient mice develop normally and are not predisposed to tumors, *p21-/-* fibroblasts exhibit abnormalities in the regulation of the cell cycle. Specifically, loss of p21 leads to a 2-4 fold elevation in the activity of the G1 cyclin-dependent kinase cdk2, a shortening of the G1 phase and a reduced G1 cell size. Furthermore, in combination with a loss of function mutation in the tumor suppressor gene retinoblastoma, loss of *p21* causes cells to proliferate under conditions that normally induce quiescence. For instance, *p21-/-;Rb-/-* fibroblasts are capable of anchorage-independent growth and proliferate at concentrations of growth factors insufficient for normal cell proliferation. Thus, although p21 is not necessary for differentiation or tumor suppression in the mouse, p21 seems to be a critical regulator of cell proliferation.

In addition, we have found that p21 is an important component of the DNA damage checkpoint. Gamma-irradiation of fibroblasts results in the accumulation of the tumor suppressor protein p53, which leads to a G1 arrest. *p21* is a transcriptional target of p53 and is upregulated following DNA damage. To determine the role of p21 in this checkpoint pathway, we analyzed the irradiation response of *p21*-deficient fibroblasts. *p21-/-* fibroblasts failed to downregulate cdk2 activity following irradiation and were impaired in their ability to arrest in G1. Thus, p21 is required for cdk2 inhibition in fibroblasts in response DNA damage.

Taken together our data indicate that p21 is a critical regulator of cdk2 that plays an important role in the regulation of the cell cycle both in response to extracellular signals as well as the DNA damage checkpoint pathway.

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Chapter 1

The Cyclin-dependent Kinase Inhibitor p21

In this thesis I am going to describe work that I have performed to understand the biological function of the Cyclin-dependent kinase inhibitor p21. This thesis consists of five chapters, including an introductory chapter, three chapters in which I describe my research and a chapter discussing future directions of this work. The first chapter is divided into four parts. In the first part, I summarize the research that led to the identification of the enzymes that regulate the cell cycle, the Cyclin-dependent kinases (CDKs). Next, I discuss the regulation of CDKs, focusing on how different regulatory mechanisms impinge on CDK structure and activity. The third part is dedicated to describing a class of CDK regulatory proteins, the Cyclin-dependent Kinase Inhibitors (CKIs). Here, I summarize information regarding CKIs in different organisms, from yeast to humans. Finally, the last part of the introduction focuses on CKIs in mammalian cells and in particular p21. This part describes the discovery and cloning of p21, the mechanisms of growth inhibition by p21 and functions that have been attributed to p21 such as p53-mediated growth suppression.

The second chapter describes the generation of chimeric mice composed of wild-type and *p21*-deficient cells. These mice were generated to address some of the functions that have been proposed for p21, including differentiation and the p53-mediated DNA damage response. Here, we report that p21 is an important p53-target in the G1 arrest response to DNA damage in fibroblasts. This chapter also reports the generation of a *p21*-deficient mouse strain.

The third chapter deals with the role of p21 in the regulation of the normal cell cycle. In addition, we describe the generation and analysis of cells deficient for two important negative regulators of cell proliferation, p21 and pRB. Based on experiments performed with *p21-/-;Rb-/-* cells we propose a model for the regulation of the cell cycle by extracellular signals.

The fourth chapter focuses on the study of the mechanism of G1 arrest in response

to DNA damage. Here, we show that p53, through p21, co-opts the normal cell cycle regulatory machinery to impose a cell cycle block following DNA damage.

Finally, the last chapter summarizes the information presented in the previous three chapters and describes future potential directions of this work.

CELL CYCLE OVERVIEW

The cell cycle is the ordered set of events that leads to the generation of two cells from a single cell. This process requires accurate replication of the genetic material precisely one time followed by orderly segregation of two complete sets of chromosomes into the daughter cells. The simplest cell cycle consists of two alternating phases, an S-phase during which the genomic DNA is replicated and an M-phase in which chromosomes are segregated. These S/M cycles occur without significant cell growth and are typical of early embryonic cycles in which the fertilized egg simply subdivides into smaller and smaller cells. The majority of cell cycles however, contain two additional intervening phases, Gap1, from the end of mitosis to the beginning of S-phase, and Gap2, from the end of S-phase to the beginning of mitosis. Cell growth primarily occurs during these gap phases which are necessary for maintaining a homogeneous cell size in a cell population.

Progression through the cell cycle is regulated by a family of protein kinases, the cyclin-dependent kinases (CDKs) (reviewed by Morgan 1997). These enzymes, which are conserved in all eukaryotes, are composed of a catalytic kinase subunit and a regulatory cyclin subunit. Whereas the number of catalytic subunits is small (in some organisms there is only one), there are invariably multiple regulatory cyclin subunits. These regulatory subunits are not only required to activate the kinase, but are also important determinants of substrate specificity. Progression through the different phases of the cell cycle is determined by the assembly of different cyclin/CDK complexes. The formation of these complexes is largely regulated by the availability of cyclins; whereas the levels of the catalytic subunit(s) are typically in constant excess, cyclin levels oscillate with the cell cycle, determining the assembly and disassembly of different cyclin/CDK complexes with different substrate specificities (reviewed by Murray 1993). This regulation ensures the orderly progression through the different phases of the cell cycle.

The identification and cloning of Cyclin-dependent kinases

The first evidence suggesting the existence of soluble cell cycle regulatory factors came from mammalian cell fusion experiments performed in 1970 (Johnson et al. 1970; Rao and Johnson 1970). In these experiments, it was found that the fusion of mitotic cells to interphase cells (in G1-, S- or G2-phases) induced mitotic events in the interphase nuclei. This results suggested the existence of a dominant transacting factor that could induce mitosis. Subsequently an activity with similar characteristics was also identified in mature frog eggs, a more useful cell type for biochemical dissection due to their large size (~1mm in diameter) (Masui and Markert 1971). Oocytes could be harvested from female frogs and stimulated to mature *in vitro* with progesterone, which induced them to enter into and subsequently arrest at meiosis awaiting fertilization. Researchers found that these mature oocytes contained an activity that could induce the maturation of other oocytes as transplantation of a small amount of cytoplasm from an unfertilized egg into an immature oocyte induced meiotic maturation (Masui and Markert 1971). This experiment indicated the existence of a cytoplasmic factor that could induce meiosis. This putative factor was termed maturation promoting factor (MPF).

Further analysis revealed two important features about MPF. First, the generation of MPF in progesterone-treated eggs could be blocked by protein synthesis inhibitors (Newport and Kirschner 1984). Second, MPF was also present during the normal cell cycle and was present at high levels at mitosis and low levels during interphase (Gerhart et al. 1984). Considered together, these experiments suggested that MPF consisted of a protein that was periodically synthesized during the cell cycle. A candidate protein with the expected oscillatory pattern was identified in sea urchin eggs during experiments designed to study protein synthesis following fertilization (Evans et al. 1983). Whereas most

proteins progressively accumulated after fertilization, the levels of one protein oscillated with the cell cycle (high at mitosis and low at interphase). This protein was biochemically purified and was called cyclin B. Cyclin B turned out to be one component of MPF and a prototype CDK-regulatory subunit.

The catalytic kinase subunit of the CDK complex (the second component of MPF), was identified in a genetic screen to find genes encoding cell cycle regulatory proteins in the yeast *S. pombe*. Following mutagenesis, strains containing conditional temperature sensitive mutations that resulted in a failure to undergo cell division at the restrictive temperature were isolated. One of these cell division cycle (cdc) mutants, $cdc2^{ts}$, was able to replicate its DNA but failed to enter mitosis at the restrictive temperature (reviewed by Murray 1993). Thus, *cdc2* was predicted to encode a protein that was necessary for entry into mitosis. The *cdc2* gene was then isolated using a complementation approach in which $cdc2^{ts}$ yeast strains were transformed with a genomic DNA library and selected for the ability to enter mitosis (i.e. grow) at the restrictive temperature. Based on sequence analysis, the *cdc2* gene was predicted to encode a protein with homology to protein kinases.

Significantly, the cloning approach used for isolation of the *cdc2* gene (complementation of the $cdc2^{ts}$ strain) was used to successfully isolate *cdc2* homologues from other organisms including *S. cerevisiae* and humans (reviewed by Murray 1993). Among the genes identified was the *S. cerevisiae CDC28* gene (Beach et al. 1982), which encoded a protein kinase with 63% sequence identity to Cdc2. Interestingly, *CDC28* was found to be essential for DNA replication, as yeast strains with a temperature sensitive mutation in *CDC28* arrested just prior to DNA synthesis when grown at the restrictive temperature. Thus, mutations in the *cdc2* and *CDC28* genes caused arrest at distinct phases of the cell cycle, with $cdc2^{ts}$ preventing entry into mitosis and $cdc28^{ts}$ blocking DNA replication.

A unified view of the regulation of mitosis was attained with the purification of MPF (Lohka et al. 1988). After years of effort, methods were developed that allowed large scale purification of MPF. It was then found that MPF had protein kinase activity and that this kinase was composed of two different subunits which were homologues of the previously identified cyclin B and Cdc2 (Gautier et al. 1988). Furthermore, it was also determined that the changes in MPF activity which occurred during the cell cycle were due to oscillations in the levels of cyclin B protein, while the levels of the Cdc2 protein remained constant (Murray and Kirschner 1989). Unexpectedly, these changes in cyclin B were not determined by periodic protein synthesis, as had been previously hypothesized, but were instead due to changes in protein stability (Murray et al. 1989). Cyclin B was synthesized throughout the cell cycle but was abruptly degraded at the end of mitosis. In addition, it was demonstrated that periodic cyclin B accumulation was sufficient to drive the oocyte cell cycle (reviewed by Murray 1993). Considered together, these experiments indicated that entry into mitosis was regulated by a heterodimeric protein kinase whose activity was determined by the presence of the regulatory cyclin subunit.

The cell cycle in other organisms is similarly regulated by heterodimeric cyclin/CDK complexes. In yeast, multiple cyclins were discovered that bound Cdc2/28 at different phases of the cell cycle. Yeast strains harboring mutations in the various cyclins arrested at different stages of the cell cycle indicating that each cyclin governed a different cell cycle transition. In mammalian cells, the regulation of the cell cycle has been found to be even more complicated involving not only multiple cyclins, but also multiple kinase subunits. The genes encoding these subunits were identified primarily by their ability to complement *cdc* mutations in yeast or by their homology to known cyclins or CDKs (Pines and Hunter 1989; Pines and Hunter 1990; Richardson et al. 1990; Koff et al. 1991; Lew et al. 1991; Matsushime et al. 1991; Tsai et al. 1991; Matsushime et al. 1992; Meyerson and Harlow 1994). In short, the somatic cell cycle in mammalian cells has been

shown to be regulated by at least four different cyclin/CDK complexes. Progression through G1 requires the activity of CDK4 or CDK6 (a CDK4 homologue) in association with D-type cyclins as well as CDK2 bound to cyclin E. CDK2 binds cyclin A during S-phase and this activity is necessary for DNA replication. Finally, cyclin B/CDC2 regulates entry into mitosis.

REGULATION OF CYCLIN-DEPENDENT KINASES

Proper regulation of the activity of CDKs is clearly vital to cell survival. There exist multiple conserved levels of regulation of CDK activities (reviewed by Morgan 1995). First, CDK activity is regulated by heterodimerization with cyclins, and therefore can be affected by processes that determine the rate of synthesis, subcellular localization and degradation rate of cyclins. Second, the activity of CDKs is regulated by activating and inhibitory phosphorylation events. Finally, CDK activity can be altered through the interaction with assembly factors, adaptors and cyclin-dependent kinase inhibitors (CKIs). These multiple levels of regulation allow tight control of cell cycle transitions and thus of cell proliferation.

The catalytic subunit

CDKs are serine/threonine protein kinases that phosphorylate substrates with the sequence Z-S/T-P-X-Z, where Z is a polar amino acid and X is generally a basic amino acid (one letter amino acid notation). The typical catalytic subunit is approximately 35-40 kDa in size and is little more than a minimal protein kinase domain. These subunits are >35% identical in primary sequence and are in an inactive conformation in their monomeric form.

The crystal structure of human CDK2 (bound to ATP) has been solved (De Bondt

et al. 1993). Analysis of this protein reveals two lobes, an N-terminal lobe and a C-terminal lobe, which are separated by the catalytic cleft. The N-terminal lobe is comprised primarily of a b-sheet but also contains a large helix called PSTAIRE which is conserved in all CDKs. The C-terminal lobe is predominantly helical and contains a large loop (the T-loop) whose position determines substrate accessibility to the catalytic site. When the kinase is in the monomeric form the T loop is in a conformation that interferes with substrate binding and the γ-phosphate of ATP is poorly positioned for phospho-transfer. Thus, the lack of kinase activity of monomeric CDKs can be attributed to steric interference for substrate binding and phosphate transfer.

CDK regulation by cyclins

The activation of CDKs requires a conformational change induced by binding of the cyclin subunit to its kinase partner. The cyclins constitute a diverse family of proteins ranging from 35-90 kDa with little amino acid sequence similarity. The cyclin family is defined according to both structural and functional criteria. First, all cyclins share a structural motif ~100 amino acids long termed the cyclin box. Mutations in the cyclin box abolish CDK binding and activation, suggesting that this domain may be involved in direct interaction with the CDK subunit. Second, cyclins are defined by their ability to form dimers with protein kinases resulting in kinase activation.

Although cyclins were originally described as cell cycle oscillatory proteins, it is now clear that the levels of some cyclins remain constant during the cell cycle. Furthermore, this family also includes proteins that are not involved in cell cycle regulation, such as p35 (Tsai et al. 1994), which is involved in neuronal differentiation.

The crystal structures of two cyclins have been solved: a truncated form of cyclin A, which lacks the N-terminus (Brown et al. 1995), and the distantly related cyclin H (Kim

et al. 1996). Both cyclins contain two helical domains formed by a bundle of 5 a-helices, an N-terminal a-helix and a C-terminal domain. The amino-terminal of the 5-helical domains corresponds to the conserved cyclin box. Whereas the tertiary structure of the two helical domains is very similar between the cyclins in these studies, the topology of the N- and C-termini diverges greatly. Thus, the structurally similar helical domains might be conserved among all cyclins, and the variable domains found outside these regions are likely to be important in determining the activity of each cyclin-CDK towards specific substrates (Andersen et al. 1997).

Additional information on the regulation of CDKs by cyclin binding comes from the crystal structure of a cyclin-CDK pair, cyclin A bound to CDK2-ATP (Jeffrey et al. 1995). Two major interfaces appear to contribute to cyclin A-CDK2 binding. The first interface is formed by helices 3 and 5 of the cyclin box and the CDK PSTAIRE helix. This interaction involves motifs that are structurally conserved in both cyclins and CDKs and is likely to be present at the core of the all cyclin-CDK quaternary structures. The second interface is formed by the N-terminal helix of cyclin A, and the C-terminal domain of CDK2. This interaction includes a structural motif of cyclin A that is not conserved among cyclins, and demonstrates that non-conserved domains contribute to the specificity of cyclin-CDK binding. In addition, this structure provides a framework for understanding the mechanism by which cyclins activate CDKs. The binding of Cyclin A to CDK2 results in conformational changes only in the CDK subunit. These changes, which lead to CDK activation, include the removal of the T-loop from the "mouth" of the catalytic site thus allowing substrate binding and the proper positioning of ATP for phospho-transfer.

CDK regulation by phosphorylation

In addition to cyclin binding, full kinase activation requires the phosphorylation of a

conserved threonine residue in the T-loop (T160 in human CDK2). The effects of phosphorylation at this site on CDK activity differ depending on the cyclin/CDK complex. Whereas phosphorylation of T160 is necessary for cyclin A binding to CDC2 and the activation of cyclin B/CDC2, it is not required for either assembly or activation of cyclin A/CDK2, although it does lead to an 80-300 fold increase in kinase activity (reviewed by Morgan 1997). The crystal structure of cyclin A/CDK2 phosphorylated at T160 has been solved (Russo et al. 1996b). Comparison with the structure of the unphosphorylated cyclin/CDK complex reveals small conformational changes, consisting of alterations in the T loop which appear to stabilize the cyclin-CDK interaction and may increase the kinase-substrate surface.

The enzyme that phosphorylates T160 has been identified. This CDK-activating kinase (CAK) was biochemically purified from multiple organisms using CDK activation as an assay. Vertebrate CAK is composed of two subunits, cyclin H and CDK7 (Fisher and Morgan 1994). This enzyme is itself a cyclin-CDK complex and can be activated through two different mechanisms: by phosphorylation of the conserved threonine residue in the T-loop, which can be catalyzed by cyclin A/CDK2 *in vitro*, or through the association with an assembly factor (Fisher et al. 1995), MAT1 (Menage A Trois 1) (Devault et al. 1995). Vertebrate CAK can be found in at least three different states *in vivo*: in a dimeric or trimeric state (bound to MAT1), associated with the nucleotide-excision repair protein ERCC2, and in a complex with the RNA polymerase II general transcription factor TFIIH (reviewed by Harper and Elledge 1998). The significance of the interaction of CAK with ERCC2 is not known. However, it has been shown that CAK associated with TFIIH can phosphorylate the C-terminal domain (CTD) of RNA polymerase II indicating that CAK may play a role in coupling transcriptional activation to the cell cycle machinery. The view that a single kinase is responsible for both CDK activation and CTD phosphorylation has recently been challenged by the finding that in *S. cerevisiae* these two

functions are carried out by two different enzymes (reviewed by Harper and Elledge 1998). However, the identification of another enzyme with CAK activity in vertebrates has proven difficult in biochemical studies. Interestingly, *Xenopus* extracts depleted of cyclin H/CDK7 had no detectable CDK-activating kinase activity (reviewed by Harper and Elledge 1998), suggesting that cyclin H/CDK7 may be the real vertebrate CAK. If so, it suggests that budding yeast has developed different enzymes to phosphorylate CDKs and the CTD of RNA polymerase II. Alternatively, another as-yet unidentified enzyme with CAK activity may exist in vertebrates in addition to cyclin H/CDK7.

CDK activity is also regulated by inhibitory phosphorylation, which occurs in vertebrates at two N-terminal residues, threonine 14 and tyrosine 15. These residues are located in a loop that contributes to the formation of the ATP phosphate-binding site. While the crystal structure of a CDK phosphorylated at these residues has not been solved, it is thought that phosphorylation of these sites could result in a change in the orientation of the ATP γ-phosphate, which would significantly impair the phosphotransfer reaction (reviewed by Morgan 1997). In contrast to phosphorylation at the T160 residue, phosphorylation at T14 and Y15 is tightly regulated. These inhibitory phosphorylations have been implicated in controlling the timing of mitosis, the DNA damage checkpoint, and possibly the regulation of cell cycle progression through G1 and S-phase (reviewed by Morgan 1997).

Assembly factors & adaptors

Three additional classes of proteins regulate cyclin-CDK activity: assembly factors, adaptors and cyclin-dependent kinase inhibitors (reviewed by Morgan 1997). Assembly factors are thought to be important in the stabilization of cyclin-CDK interactions. In vertebrate cells, only one assembly factor has been characterized, MAT1 (Menage A Trois

1) (Devault et al. 1995). This protein was biochemically purified from *Xenopus* extracts in association with cyclin H/CDK7 (Devault et al. 1995). Using a reverse genetics approach, *MAT1* was cloned and found to encode a RING finger protein that is necessary to stabilize complexes containing *in vitro* translated CDK7 and cyclin H (Tassan et al. 1995). The existence of another assembly factor in mammalian cells has been inferred from experiments in fibroblasts overexpressing cyclin D. Whereas overexpression of cyclin D in normal fibroblasts leads to the formation of cyclin D-CDK4 (CDK6) complexes, this does not occur in serum-starved fibroblasts, which suggests the existence of a serum-dependent factor necessary for the assembly or stability of these complexes (Matsushime et al. 1994).

Adaptors are typically 9-18 kDa proteins that bind tightly to CDKs and are important for their function (reviewed by Pines 1996). They are functionally conserved between yeast and humans, and are thought to target CDKs to substrates or regulatory proteins. The crystal structure of the adaptor protein CKS1 bound to CDK2 has been derived, and illustrates a possible mechanism for how adaptors may function (Bourne et al. 1996). CKS1 binds to the C-terminal lobe of CDK2, away from the catalytic cleft and the cyclin binding site. This interaction does not affect CDK2 conformation. However, CKS1 binding may contribute to substrate recognition, as the protein contains a positively charged pocket which is oriented in the same plane as the catalytic site, that may serve to target the CDK to phospho-substrates. Notably, this pocket domain is conserved in other CKS proteins.

CYCLIN-DEPENDENT KINASE INHIBITORS

Cyclin-dependent kinase inhibitors (CKIs) are proteins that bind to CDKs and block their catalytic activity (reviewed by Peter 1994). CKIs have been described in organisms

ranging from yeast to humans, and are involved in a wide variety of processes. They have been implicated in functions such as the regulation of cell cycle progression and the integration of signal transduction pathways with the cell cycle machinery.

S. Pombe: Rum1

In yeast, CKIs have been demonstrated to act in the regulation of cell cycle transitions (both G1/S and G2/M) as well as in metabolic and cell-cell signaling pathways (reviewed by Mendenhall 1998). In *S. pombe* only one CKI has been identified to date, Rum1 (replication uncoupled with mitosis 1). *Rum1* was identified in a screen for genes that when overexpressed led to extra-rounds of DNA replication in the absence of mitosis (Moreno and Nurse 1994). When cloned this gene was found to encode an inhibitor of the mitotic CDK, Cdc13/Cdc2 (Correa-Bordes and Nurse 1995). Consistent with this, inactivation of *cdc13* results in endoreduplication, the same phenotype as *rum1* overexpression. In addition to its mitotic function, Rum1 can also inhibit the G1 CDK, Cig2/Cdc2 (Correa-Bordes and Nurse 1995). Specifically, Cig2/Cdc2 inhibition by Rum1 early in G1 is necessary for a pre-START G1-phase (G1-phase prior to commitment to cell division), which is essential for mating as well as proper metabolic control. Consistent with this role, *rum1-Δ* results in a shortened G1-phase, decreased fertility and lack of proper arrest in the absence of a nitrogen source (Moreno and Nurse 1994). This phenotype can be partially suppressed by deletion of the G1 cyclin, *cig2* (Correa-Bordes and Nurse 1995). Finally, cells deficient for both *Rum1* and genes required for S-phase entry (*Cdc10* or *Cdc18*) undergo cell division without DNA replication, further underscoring the importance of Rum1 in the regulation of entry into mitosis (Correa-Bordes and Nurse 1995).

Cyclin-dependent kinase inhibitors in *S. cerevisiae*:

- **Pho81**

Three CKIs have been described in *S. cerevisiae*: Pho81, Sic1 and Far1. Pho81 is important for the regulation of phosphate metabolism in budding yeast. *S. cerevisiae* requires inorganic phosphate (P_i) for growth and division. In an environment with low concentrations of P_i, cells induce the expression of genes coding for phosphatases and P_i transporters, which serve to increase intracellular levels of P_i. These changes are mediated by Pho4, a transcriptional activator that is regulated in response to P_i through the activity of the Pho80/Pho85 protein kinase. When P_i is present at high concentrations, Pho80/Pho85 phosphorylates Pho4, which abolishes Pho4 transcriptional activation. Pho80/Pho85 is classified as a cyclin/CDK based on structural homology although it has no apparent role in cell cycle regulation. This kinase is regulated by Pho81, a CKI which is activated at low concentrations of P_i (reviewed by Mendenhall 1998). *PHO81* is the first CKI that was cloned, and it encodes a large protein of 134kDa with 6 ankyrin repeats. These structural motifs are present in a wide variety of proteins and are thought to play a role in protein-protein interactions. *In vitro*, Pho81 binds to Pho80 (cyclin subunit) alone or in association with Pho85, which results in kinase inhibition. Thus, induction of Pho81 in response to low intracellular concentrations of P_i leads to Pho85/Pho80 inhibition and increased expression of Pho4 responsive genes coding for enzymes that restore intracellular P_i levels.

- **Sic1**

In *S. cerevisiae*, both G1 and S-phase cyclins are transcribed at the same time during G1. Sequential activation of G1 and S-phase cyclin/CDK complexes is accomplished by

initially maintaining S-phase cyclin/CDKs in an inactive state (Schwob et al. 1994). This is achieved by the cyclin-dependent kinase inhibitor, Sic1 (Mendenhall 1993). *SIC1* encodes a protein of ~40 kDa that specifically inhibits S-phase kinases, Cdc28/Clbs (Mendenhall 1993; Schwob et al. 1994). This inhibitor is synthesized during G1 and inactivates newly assembled Cdc28/Clb complexes prior to S-phase (Mendenhall 1993). Consistent with this, *sic1-D* results in the premature activation of S-phase CDKs and consequently G1 shortening (Schwob et al. 1994). The appropriate activation of Cdc28/Clbs/Sic1 complexes in late G1 occurs by phosphorylation of Sic1. This is accomplished by the G1 CDKs (Cdc28/Clns) and targets Sic1 for degradation through the ubiquitin-proteosome pathway (hence the name of Sic1, substrate and inhibitor of Cdc28) (Nugroho and Mendenhall 1994). Surprisingly, the only essential role of the G1 CDKs is to inactivate Sic1. Whereas yeast strains deficient for all three G1 cyclins are inviable, deletion of *SIC1* suppresses this phenotype (Schneider et al. 1996; Tyers et al. 1996). These data indicate that the Sic1 inhibitor is an important regulator of the G1/S transition in *S. cerevisiae.*

- **Far1**

Haploid *S. cerevisiae* cells exist in two mating types, MATa and MATa, which can conjugate to form a diploid cell. This mating process is initiated by the secretion of a peptide hormone called α-factor which triggers the expression of genes necessary for fusion of neighboring cells of opposite mating types. Such changes in gene expression are induced by the STE12 transcription factor, which is activated by phosphorylation in response to pheromone treatment. Pheromone binding to the transmembrane receptor results in the activation of a MAPK pathway and the phosphorylation of STE12 (reviewed by Peter 1994). Far1, is a STE12 responsive gene that is required to orient cell growth in the direction of the pheromone source. In addition, Far1 is also essential to synchronize the

cells in the cell cycle so that the deleterious effects of cell fusion in two different stages of the cycle are avoided. Synchrony is accomplished by imposing a cell cycle block at START (Chang and Herskowitz 1990), which is mediated through the direct inhibition of the G1 CDK, Cdc28/Cln2 (Chang and Herskowitz 1990; Peter and Herskowitz 1994). In fact, Far1 (Factor arrest resistant1) was originally identified in a screen for mutations that prevented cells from arresting in the presence of pheromone (Chang and Herskowitz 1990). Significantly, *far1-Δ* strains are not only impaired in their pheromone response but also in their normal cell cycle control exhibiting a shorter G1-phase (McKinney et al. 1993). These data indicate that Far1 is also an important regulator of G1 CDKs during the normal cell cycle.

CKIs in *Drosophila melanogaster*: Dacapo

Dacapo (*DAP*), the *Drosophila* cyclin-dependent kinase inhibitor, was isolated by two different groups in an enhancer trap screen (Lane et al. 1996) and in a modifier screen (Nooij et al. 1996). *Dacapo* encodes a protein of 245 amino acids that has inhibitory activity on cyclin E/Cdk2 (Lane et al. 1996; Nooij et al. 1996) but not on cyclin A/Cdc2 or cyclin B/Cdc2 (Lane et al. 1996).

Several lines of evidence suggest that DAP may be implicated in the regulation of cell cycle withdrawal upon terminal differentiation in the *Drosophila* embryo. First, the pattern of expression of *dap* mRNA in the embryo correlates with cells undergoing terminal differentiation (Lane et al. 1996; Nooij et al. 1996). Second, in transgenic models, *dap* overexpression is sufficient to block cell proliferation (Lane et al. 1996; Nooij et al. 1996). Third, *dap/dap* mutant flies inappropriately divide for an extra cycle during development, which results in tissue hypercellularity (Lane et al. 1996; Nooij et al. 1996). Interestingly, homozygous *dap* mutant cells only proliferate for a single extra cell cycle and

then arrest, suggesting that DAP may be important for establishing this arrest but not for its maintenance (Lane et al. 1996; Nooij et al. 1996). Taken together, these data indicate that DAP is required for the timely cell cycle withdrawal that accompanies terminal differentiation in *Drosophila* embryos. In addition, DAP must be important in other developmental processes, as mutation in this gene results in embryonic lethality (Lane et al. 1996; Nooij et al. 1996).

Finally, like the yeast inhibitors, DAP does not seem to be involved in the DNA damage checkpoint. *Dap* expression does not change following 40 Gy of X-rays, a dose sufficient to cause a significant number of chromosome breaks (Nooij et al. 1996). Thus, CKIs in invertebrates do not appear to function as part of the DNA damage checkpoint.

Xenopus laevis CKIs: $p27^{XIC1}$ & $p28^{KIX1}$

Two genes encoding CKIs have been reported to date in *Xenopus*, $p27^{XIC1}$ (Su et al. 1995) and $p28^{KIX1}$ (Shou and Dunphy 1996). These genes were cloned using a degenerate PCR based assay with primers designed to hybridize to sequences in human and mouse CIP/KIP inhibitors (see below). $p27^{XIC1}$ and $p28^{KIX1}$ share 90% identity in their amino acid sequence and contain an N-terminal CDK binding domain that is highly similar to CIP/KIP inhibitors (~40% identical) (reviewed by Hengst and Reed 1998). *In vitro*, both $p27^{XIC1}$ and $p28^{KIX1}$ inhibit cyclin E/CDK2 and cyclin A/CDK2 but not cyclin B/CDC2 (Su et al. 1995; Shou and Dunphy 1996). In addition to the conserved CDK binding domain, these inhibitors also contain a C-terminal domain that interacts with proliferating cell nuclear antigen (PCNA) (Su et al. 1995; Shou and Dunphy 1996). PCNA is an auxiliary factor of DNA polymerase δ, and it is necessary for DNA replication and DNA repair. *In vitro*, $p27^{XIC1}$ inhibits DNA replication in *Xenopus* extracts in a PCNA dependent manner (Su et al. 1995). Finally, both inhibitors contain

consensus CDC2 phosphorylation sites and appear to be phosphorylated by this kinase *in vitro* (Su et al. 1995; Shou and Dunphy 1996), although the significance of this is presently unclear.

Expression studies have suggested that $p28^{KIX1}$ may be implicated in terminal differentiation (Shou and Dunphy 1996). The mRNA levels of this inhibitor are upregulated > 20 fold shortly after gastrulation when some cell types undergo a permanent cell cycle arrest. Interestingly, in contrast to DAP, the levels of this inhibitor remain high in somatic lineages, suggesting that $p28^{KIX1}$ may have a role in the maintaining the cell cycle arrest associated with terminal differentiation.

CYCLIN-DEPENDENT KINASE INHIBITORS IN MAMMALIAN CELLS

In contrast to our understanding of most areas in cell biology, the understanding of CKIs has developed further in mammalian cells than in other organisms. Mammalian CKIs have been implicated in a wide array of processes, including coupling signal transduction pathways downstream of cytokines and hormone receptors to the cell cycle machinery, the DNA damage checkpoint, differentiation and tumor suppression. In contrast to the situation in lower eukaryotes, these mammalian inhibitors seem to play a limited role in the regulation of intrinsic cell cycle events. Mammalian CKIs are classified into two structurally and functionally distinct families: the INK4 family and the CIP/KIP family. Whereas members of the CIP/KIP (CDK interacting protein/CDK inhibitory protein) family inhibit a broad range of cyclin/CDK complexes, INK4 inhibitors (inhibitors of CDK4) (reviewed by Carnero and Hannon 1998) are specific for CDK4 or the CDK4 homologue, CDK6. CDK4 is an extremely important CDK in the regulation of cell proliferation (see below). Thus, it is not surprising that there would be a whole family of inhibitors specifically dedicated to CDK4 regulation.

CDK4(6) is one of two CDKs that regulate progression through the G1-phase of the mammalian cell cycle and commitment to the cell division cycle (reviewed by Sherr 1994). In conjunction with CDK2, CDK4 determines whether a cell will enter into S-phase and divide. CDK4 acts in G1 before CDK2 and is activated in response to growth factors (Matsushime et al. 1991). Growth factor signaling, through the activation of the ras pathway, results in transcriptional upregulation of the CDK4 activators, the D-type cyclins. These cyclins target CDK4 to its substrate, the retinoblastoma protein, pRB (Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993). pRB is a tumor suppressor that is thought to act by blocking progression through the G1-phase of the cell cycle (reviewed by Weinberg 1995). The growth suppressive properties of pRB are inactivated by phosphorylation (reviewed by Weinberg 1995). CDK4 is necessary for pRB inactivation. Furthermore, the only essential function of CDK4 for proliferation appears to be to phosphorylate pRB as suggested by experiments that showed that inactivation of cyclin D/CDK4 with neutralizing antibodies against cyclin D1 only arrests cells that contain functional pRB (Lukas et al. 1995a; Lukas et al. 1995b). The importance of the Cyclin D-CDK4-pRB pathway in the regulation of proliferation is underscored by the fact that some component of this pathway is mutated in the vast majority of tumors (reviewed by Hirama and Koeffler 1995; reviewed by Sherr 1996).

INK4 INHIBITORS

The INK4 family consists of $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$ (reviewed by Carnero and Hannon 1998). These proteins are >30% identical in their primary sequence and are comprised of 4-5 ankyrin repeats. These repeats are structural motifs (helix-β-turn-helix) that are involved in protein-protein interactions and have been described in a wide variety of proteins, including the yeast CKI Pho81.

- **$p16^{INK4a}$**

The first INK4 that was discovered is $p16^{INK4a}$. p16 was first noted in a study to determine the effects of oncogenic transformation on the CDKs (Xiong et al. 1993b). In this study, it was found that transformation of normal human fibroblasts with the viral oncogene Large T Antigen resulted in the rearrangement of the protein components of several cyclin-CDK complexes, including cyclin D/CDK4. While in normal cells, CDK4 was primarily found associated with D-type cyclins, following transformation it appeared almost exclusively associated with a protein of 16 kDa. Using CDK4 as a "bait" in a yeast two hybrid protein-protein interaction screen, the gene encoding p16 was cloned (Serrano et al. 1993). The *p16* gene encodes a protein of 146 amino acids that is almost exclusively composed of 4 ankyrin repeats. NMR studies have revealed that p16 is a highly a-helical protein (Tevelev et al. 1996; Byeon et al. 1998). p16 binds to CDK4 (or CDK6) *in vitro* but not to other CDKs (Serrano et al. 1993). In addition, recombinant p16 inhibits active cyclin D/CDK4 complexes (preassembled in insect cells) (Serrano et al. 1993). This inhibitory activity was confirmed *in vivo*; overexpression of p16 results in CDK4 inhibition and a subsequent cell cycle arrest in the G1-phase of the cell cycle (Serrano et al. 1995). As expected for a cell cycle arrest that is imposed through CDK4 inhibition, this arrest is dependent on the presence of functional pRB (Koh et al. 1995; Lukas et al. 1995a; Medema et al. 1995).

Several lines of evidence suggest that p16 is a tumor suppressor. First, p16 is a cell cycle inhibitor and p16 overexpression is sufficient to stop cell proliferation (Serrano et al. 1995 et al.). Second, and more importantly, p16 is frequently inactivated in human cancer (Nobori et al. 1994). Furthermore, Kamb et al., identified the *p16* gene as the candidate tumor suppressor gene in 9p21, a chromosomal region that is altered in a large number of

tumors including familial melanoma (Kamb et al. 1994). Finally, targeted disruption of *p16* in the mouse results in tumorigenesis (Serrano et al. 1996). *p16-/-* mice develop lymphomas and sarcomas at an early age, and this predisposition is enhanced by exposure to carcinogens (Serrano et al. 1996).

The concept of p16 as a tumor suppressor protein was confounded by the discovery that the *p16* locus encodes for a second protein with growth suppressive properties, p19 ^{ARF}(Alternative Reading Frame) (Quelle et al. 1995b). p19 ^{ARF} is structurally and functionally unrelated to p16 but is partially encoded from the *p16* coding sequence in an alternative reading frame. Like *p16*, *p19^{ARF}*, induces growth arrest when overexpressed, albeit through a different mechanism involving stabilization of the p53 tumor suppressor protein (Pomerantz et al. 1998; Zhang et al. 1998). Due to overlap in coding sequences, most mutations that inactivate *p16* in tumors also affect *p19 ^{ARF}*(Hirama and Koeffler 1995). Knock-out mice have been generated which are deficient for p19 ^{ARF} but retain an intact *p16* gene. Interestingly, these animals exhibit the same tumor predisposition as the mice described earlier, which were deficient for both p16 and p19 ^{ARF} (Kamijo et al. 1997), suggesting that loss of p19 ^{ARF} alone could account for the tumor phenotype. The question of whether p16 is a tumor suppressor remains to be solved (reviewed by Haber 1997), but some insight into this question could be gained by examining the tumor predisposition of mice exclusively deficient for p16. It is possible that both *p16* and *p19 ^{ARF}* are tumor suppressor genes, which would potentially explain the high frequency of mutations affecting this locus in cancer.

- **p15^{INK4b}**

The *p15^{INK4b}* gene was cloned by screening a human cDNA library at low stringency with the coding sequence of *p16* (Hannon and Beach 1994). p15 is related to p16 in many

ways; these proteins are 70% identical in their primary sequence and are almost exclusively composed of four ankyrin repeats. In addition, the *p15* gene is closely linked to *p16* and as a consequence, tumor deletions affecting *p16* often result in the deletion of *p15* (Hirama and Koeffler 1995).

Despite the relatedness of p15 to p16, these proteins are implicated in very different biological processes. First, the pattern of expression of these genes in the mouse is very different; *p15* is expressed broadly in many tissues while *p16* expression is limited to the spleen and the lung (Quelle et al. 1995a). Second, p15 is specifically involved in TGFβ signaling (Hannon and Beach 1994). TGFβ is a multifunctional cytokine that elicits a p15-dependent cell cycle arrest in a cell-type-specific manner. In response to TGFβ, the *p15* gene is transcriptionally upregulated 30 fold resulting in increased p15 binding to CDK4 and CDK4 inhibition (72). This binding occurs in the cytoplasm and precludes the binding of another CKI, p27, in the nucleus (Reynisdottir and Massague 1997). This results in increased pools of free p27 that is available to bind to and inhibit CDK2. Thus, TGFβ treatment results in the inhibition of both CDK4 and CDK2 kinases. Considered together these experiments suggest that p15 and p16 are likely to be important in different processes.

- **$p18^{INK4c}$ & $p19^{INDK4d}$**

Two other INK4 inhibitors have been isolated: $p18^{INK4c}$ and $p19^{INK4d}$. The genes encoding these inhibitors were isolated using yeast two hybrid screens. *p18* was cloned in a search for cDNAs encoding CDK4 (Hirai et al. 1995) and CDK6 (Guan et al. 1994) interacting proteins. The *p19* gene was identified in the same search for genes coding for CDK4 (Hirai et al. 1995) binding proteins and independently by a group looking for genes encoding binding partners of the orphan steroid receptor Nur77 (Chan et al. 1995). p18

and p19 are 40% identical and contain five tandemly repeated ankyrin motifs; these proteins are also very similar to the other INK4 inhibitors.

Recombinant p18 and p19 bind CDK4 (and CDK6) both in a monomeric state as well as bound to D-type cyclins resulting in inhibition of kinase activity (Guan et al. 1994; Chan et al. 1995; Hirai et al. 1995). However, *in vivo*, these inhibitors are exclusively found associated with monomeric CDK4 or CDK6 at the expense of D-type cyclins (Guan et al. 1994). As with other INK4 inhibitors, growth arrest by p18 and p19 is also dependent on the presence of functional retinoblastoma protein (Guan et al. 1994).

Despite the overall conservation of the INK4 inhibitors, they are involved in very different biological processes. Whereas p15 and p16 are candidate tumor suppressors and their loci are frequently altered in tumors, p18 and p19 are rarely mutated in tumors (Zariwala et al. 1996; Zariwala and Xiong 1996). Unlike the other INK4 inhibitors, p18 seems to play a role in differentiation, and its levels are upregulated 10-50 fold in cells differentiating into the myocyte or adipocyte lineages (Phelps et al. 1998). Finally, p19 may regulate intrinsic cell cycle processes, as the levels of this inhibitor oscillate during the cell cycle (Hirai et al. 1995).

CIP/KIP INHIBITORS

The CIP/KIP family of inhibitors includes $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (reviewed by Hengst and Reed 1998). These inhibitors differ from the INK4 inhibitors in their structure, mechanism of inhibition and specificities. CIP/KIP proteins have a conserved N-terminal domain of ~ 65 amino acids which is sufficient for CDK inhibition, and a non-conserved C-terminal domain of variable length which contains a nuclear localization signal. In contrast to INK4 inhibitors, CIP/KIP inhibitors do not bind monomeric CDKs and only interact efficiently with cyclin/CDK complexes. In addition, CIP/KIPs have a much

broader specificity than INK4 inhibitors, in that they interact with cyclin D/CDK4 (6), cyclin E/CDK2, cyclin A/CDK2 and cyclin B/CDC2.

The conserved N-terminal domain contains a CDK binding region and a cyclin binding motif. The cyclin binding motif has a consensus sequence of ZRXLFG where Z is a basic amino acid or cysteine and X is typically a basic amino acid (Zhu et al. 1995; Adams et al. 1996; Chen et al. 1996a; Lin et al. 1996). Peptides containing this sequence efficiently bind to cyclin E or A whether alone or in complex with CDK2 (Adams et al. 1996; Chen et al. 1996a). Significantly, this motif has been identified in proteins other that CIP/KIPs including p107 (Zhu et al. 1995), p130 (Adams et al. 1996), E2F1 (Adams et al. 1996) and CDC25A (Saha et al. 1997). With respect to cyclin A/CDK2 binding, these proteins fall into three categories: substrates, adaptors and regulators. The p107 and p130 pRB family members have been shown to be substrates for cyclin A/CDK2. The E2F1 protein forms a heterodimeric transcription factor with DP1 and acts as an adaptor, bringing cyclin A/CDK2 in close proximity to its substrate DP1. The CDC25A phosphatase is an activator of cyclin A/CDK2 and cyclin E/CDK2. These cyclin binding motifs are necessary for the association of these proteins with cyclin A/CDK2 (Adams et al. 1996; Chen et al. 1996a). Furthermore, short peptides spanning the cyclin binding motif are able to block each of these interactions (Adams et al. 1996; Chen et al. 1996a). Thus, one mechanism of cyclin/CDK inhibition by p21 may be to block binding to substrates and activators.

Further information on the conserved N-terminal CKI domain has been provided by the crystal structure of the N-terminal CKI domain of p27 (residues 28-96) bound to cyclin A/CDK2 (Russo et al. 1996a). This fragment has previously been shown to be both necessary and sufficient for cyclin-CDK binding and inhibition (Luo et al. 1995). This domain crystallized as a non-globular extended structure interacting with a large surface area of cyclin A-CDK2. This area is distributed between cyclin A (40%) and CDK 2

(60%) and is significantly larger than the interface between the cyclin and the CDK. p27 uses a three stage approach to binding and inhibiting cyclin A/CDK2. First, the cyclin binding motif, which forms a rigid coil, is inserted into a shallow groove in the cyclin box. This interaction, which involves motifs that are conserved in CIP/KIPs and cyclins A, D, E and B, occurs without a conformational change in the cyclin and is the first to occur, based on biochemical studies (Hall et al. 1995). Second, an amphipathic β-strand of p27 displaces and replaces the first b-strand of the b-sheet in the C-terminus of CDK2. This causes a conformational change that destabilizes ATP binding. Third, p27 inserts a tyrosine residue that is conserved in all CIP/KIPs inside the catalytic cleft. This residue mimics some interactions of the ATP base and blocks binding to ATP. Thus, p27 inhibits the catalytic activity of cyclin A/CDK2 by inducing conformational changes that affect ATP binding. In addition, CIP/KIPs can also inhibit CDKs by blocking activation by CAK (Kato et al. 1994; Polyak et al. 1994a; Aprelikova et al. 1995). The crystal structure illustrates how this might occur. Because the truncated C-terminus of p27 crystallizes in close proximity to the residue T160, which is phosphorylated by CAK, it is likely that p27 binding would interfere with CAK access to T160.

p27

p27 was originally identified as a CDK2 inhibitory activity in extracts from TGFβ treated cells (Polyak et al. 1994a). This inhibitor was purified biochemically using a cyclin E/CDK2 affinity column and cloned using information derived from protein microsequences (Polyak et al. 1994b). p27 was also cloned independently by a second group using a yeast two hybrid screen to identify proteins that interacted with cyclin D1/CDK4 (Toyoshima et al. 1994).

p27 contains a conserved N-terminal CDK inhibitory domain and a C-terminal

domain which contains a CDK consensus phosphorylation site (Polyak et al. 1994b; Toyoshima et al. 1994), which is conserved in p57 but not in p21 (Lee et al. 1995; Matsuoka et al. 1995). *In vitro*, p27 efficiently binds and inhibits cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2 (Hengst et al. 1994; Polyak et al. 1994b; Toyoshima et al. 1994). In addition, p27 also interacts with cyclin B/CDC2, albeit with a lower affinity (Toyoshima et al. 1994). *In vivo*, p27 is found associated with cyclin/CDK complexes containing CDK4 (Toyoshima et al. 1994) and CDK2 (Polyak et al. 1994a; Polyak et al. 1994b) but not CDC2 (Toyoshima et al. 1994). Surprisingly, active cyclin D/CDK4 complexes containing p27 can be found in lysates from mammalian cells (Reynisdottir and Massague 1997). Cyclin D/CDK4 may act as a reservoir for p27 regulating the amount of free p27 that is able to act on CDK2 (Reynisdottir and Massague 1997). Under various circumstances an increase in p27 bound to CDK2 is observed without a significant increase in p27 proteins levels (Zhu et al. 1996; Reynisdottir and Massague 1997). This occurs by decreasing the amount of cyclin D/CDK4 complexes that are available for p27 binding through the downregulation of cyclin D protein levels or the binding of INK4 inhibitors which preclude p27 binding (Zhu et al. 1996; Reynisdottir and Massague 1997; reviewed by Assoian 1997).

p27 may mediate a cell cycle arrest in response to multiple antimitogenic signals. p27 protein levels are induced in response to differentiation and antimitogenic factors such as vitamin D3 (reviewed by Hengst and Reed 1996), lovastatin (Hengst et al. 1994; reviewed by Hengst and Reed 1996) and rapamycin, (Nourse et al. 1994) as well as tissue culture conditions that induce quiescence such as confluency (Polyak et al. 1994a; Hengst and Reed 1996) and serum deprivation (Slingerland et al. 1994; Coats et al. 1996). These changes are largely posttranscriptional and are primarily mediated through changes in protein stability, (Pagano et al. 1995) although changes in translational efficiencies have also been documented (reviewed by Hengst and Reed 1996). p27 may also play a role in

the regulation of the normal cell cycle as its levels oscillate in some cell types with the cell cycle (Hengst et al. 1994; reviewed by Hengst and Reed 1996). In addition, p27 associates differently with cyclin/CDK complexes depending on the cell cycle stage (Soos et al. 1996).

To test the role of p27 in differentiation and the antimitogenic response, the *p27* gene was inactivated by homologous recombination in the mouse (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). *p27-/-* mice were viable and larger in size than isogenic littermate controls (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). This increase in size occurred in the absence of appreciable changes in the growth promoting hormones Insulin Growth Factor I and Growth Hormone (Kiyokawa et al. 1996) and correlated with increased tissue cellularity (Fero et al. 1996; Nakayama et al. 1996), which was particularly pronounced in tissues that normally express high levels of p27 such as the thymus and the spleen, suggesting that this phenotype is cell autonomous (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). Tissue cellularity depends on the ratio of cell proliferation to cell death. The increase in cell number in *p27-/-* mice occurred without detectable changes in cell death and was accompanied by increased number of cells incorporating the thymidine analogue BrdU, suggesting that it was due to increased cell proliferation (Kiyokawa et al. 1996). Some defects in differentiation were also observed in various cell types in the *p27-/-* mice. For example, granulosa cells from the ovary failed to differentiate into progesterone-secreting luteal cells following ovulation (Kiyokawa et al. 1996). These cells normally express high levels of *p27* and are necessary to secrete progesterone which prepares the uterine epithelium for implantation. Failure in this process resulted in female sterility. In addition, oligodendrocytes from *p27-/-* mice failed to differentiate properly in tissue culture, a phenotype that correlated with increased cell proliferation (Casaccia-Bonnefil et al. 1997). Hence, p27 seems to be required for the normal cell cycle withdrawal that is necessary for proper terminal differentiation in some

cell types.

Other data indicated that p27 mediates a cell cycle arrest in response to antimitogenic signals. For instance, the inhibition of *p27* expression in 3T3 fibroblasts with antisense oligonucleotides prevented a normal cell cycle arrest following mitogen deprivation (Coats et al. 1996). The availability of cells from *p27-/-* mice allowed direct testing of the role of p27 in response to a variety of antimitogenic stimuli. While T cells derived from *p27-/-* mice did exhibit 2-3 fold higher levels of cyclin E/CDK2 activity relative to normal cells, (Fero et al. 1996) these cells arrested normally in response to TGFβ and rapamycin, indicating that p27 is not required for a TGFβ or rapamycin-induced arrest (Nakayama et al. 1996). In addition, mouse embryo fibroblasts deficient for *p27* were largely normal and effectively arrested both at high cell densities and low serum concentrations (Nakayama et al. 1996). These results underscore the importance of gene targeting studies in the assessing of the biological function of a gene product.

In addition to causing generalized hyperplasia, loss of p27 in the knock-out mice, resulted in a predisposition to tumorigenesis (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). *p27-/-* mice developed retinal dysplasia and intermediate lobe pituitary tumors. Significantly, intermediate lobe pituitary tumors, which are rare in the mouse, are also observed in mice which lack one allele of the tumor suppressor gene *Rb* (Clarke et al. 1992; Jacks 1992; Lee et al. 1992). Although pituitary tumors appearing in *Rb+/-* mice are more invasive, the cell type of origin in both strains seems to be the same, suggesting that p27 and pRB may act in the same growth control pathway in this cell type. These data support the idea that *p27* is a tumor suppressor gene in the mouse. It is unclear whether *p27* is a tumor suppressor gene in humans as *p27* mutations are rarely detected in human neoplasias (Hirama and Koeffler 1995). Nevertheless, p27 levels are significantly reduced in some tumors, and this correlates with poor prognosis, (Porter et al. 1997) indicating that p27 loss may play some role in tumor development in humans.

p57

The *p57* cDNA was isolated using two different approaches: a low stringency screen of a mouse embryo cDNA library designed to isolate *p21* related genes (Lee et al. 1995) and in a yeast two hybrid screen for cyclin D1 interacting proteins (Matsuoka et al. 1995). These screens led to the isolation of three different *p57* cDNAs corresponding to two splice variants which differed by 13 N-terminal codons.

The p57 protein can be divided into four different regions: the characteristic N-terminal CKI domain, a C-terminal domain with limited homology to p27, and two internal domains (Lee et al. 1995; Matsuoka et al. 1995). Whereas the N- and C-terminal domains are conserved between humans and mice, the internal domains are not (Matsuoka et al. 1995), suggesting that they may not be critical for protein function. In both *in vitro* and *in vivo* studies, p57 has been found to bind to cyclin D/CDK4 (6), cyclin E/CDK2, and cyclin A/CDK2. Overexpression of p57 results in cell cycle arrest in the G1, which is independent of pRB (Lee et al. 1995; Matsuoka et al. 1995).

Like the *Drosophila* CKI *dacapo*, the pattern of expression of *p57* is suggestive of a role in differentiation. In the mouse, *p57* is expressed in terminally differentiated cells of skeletal muscle, brain, heart and eye (Matsuoka et al. 1995). In humans, it has been suggested that p57 may function as a tumor suppressor protein. The human *p57* maps to 11p15.5 (Matsuoka et al. 1995), a chromosomal region frequently rearranged in sporadic tumors. However, no *p57* mutations have been detected in sporadic tumors. The 11p15.5 region is also altered in patients with Beckwith-Wiedemann syndrome (BWS), a congenital syndrome that is characterized by a wide variety of growth abnormalities including macroglossia (enlarged tongue), gigantism and other skeletal abnormalities, visceromegalia, renal dysplasia, adrenal cytomegaly and defects in the abdominal wall

which are present with variable penetrance. Genetic analysis was performed to screen for mutations in the *p57* gene in patients with BWS. Some loss of function mutations were discovered in a subset of BWS patients, suggesting that loss of p57 may predispose to BWS (Hatada et al. 1996). Consistent with this idea, both the putative BWS gene and *p57* are imprinted (Hatada et al. 1996).

To determine the role of p57 in BWS, a mouse strain harboring a disruption of the *p57* gene was generated (Yan et al. 1997; Zhang et al. 1997). *p57-/-* or *p57+/-^{m}* (with an imprinted wild-type allele) mice exhibited some features typical of BWS including renal dysplasia, skeletal abnormalities and abdominal wall defects (Zhang et al. 1997), suggesting that loss of p57 may be involved in the development of BWS. However, *p57-/-* mice did not exhibit other features typical of BWS such as macroglossia (present in >90% of BWS patients) or somatic overgrowth (present in >50% of BWS patients) (113, 114). This difference could be attributed to species differences or alternatively, may indicate that other genes in addition to *p57* contribute to BWS (Eggenschwiler et al. 1997). In addition, *p57-/-* mice had severe gastro-intestinal defects (that were likely to be responsible for their death shortly after birth) a phenotype not seen BWS patients (Yan et al. 1997; Zhang et al. 1997; reviewed by Swanger and Roberts 1997). Histological analysis of tissues from *p57-/-* mice also revealed both increased cell proliferation and cell death (Yan et al. 1997; Zhang et al. 1997) in multiple tissues including the lens (Zhang et al. 1997). This phenotype is characteristic of *Rb-/-* embryos, and is thought to result from failure to differentiate (Morgenbesser et al. 1994). These data may suggest that p57, together with pRB, is important for differentiation of cells in the lens. Considered together these data suggest that p57 is important to implement a cell cycle arrest during differentiation of some cell types and to protect from tumor development.

THE CIP/KIP INHIBITOR p21

The p21 protein was first noted in a study by Xiong et al., which characterized proteins that associated with D-type cyclins in the WI38 human fibroblast cell line (Xiong et al. 1992). Cyclin D1 immunoprecipitates from ^{35}S-methionine labeled extracts contained various CDKs, PCNA, and a protein with an apparent molecular weight of 21 kDa, which was termed p21 (Xiong et al. 1992). A protein with a similar molecular weight was also found in CDK2 immunoprecipitates (Xiong et al. 1992). Partial V8 protease mapping was performed to compare the identity of these proteins. The peptide patterns of both 21 kDa proteins were the same, suggesting that the protein that was present in cyclin D1 immunoprecipitates was also associated with CDK2 (Xiong et al. 1992).

In a follow-up study, it was found that transformation of fibroblasts with the SV40 viral oncogene Large T Antigen resulted in the dramatic reorganization of cyclin/CDK/p21/PCNA complexes (Xiong et al. 1993b). In cells expressing Large T Antigen, CDK4 exclusively associated with a protein of 16 kDa (which turned out to be p16^{INK4a}), while p21 and PCNA were no longer found in CDK2 immunoprecipitates (Xiong et al. 1993b). These changes were not limited to oncogenic transformation by SV40 Large T Antigen, but were also observed in cells transformed with other oncogenes from adeno- and papilloma viruses (Xiong et al. 1993b). These results suggested that the rearrangement in the subunit composition of cyclin/CDK complexes following oncogenic transformation could be a generalized phenomenon. Furthermore, fibroblasts from patients with Li-Fraumeni disease, a inherited cancer syndrome, also exhibited reorganized cyclin/CDK complexes which lacked p21 (Xiong et al. 1993b). Taken together, these data suggested that p21 is an important regulator of normal cell proliferation.

p21 was purified biochemically from cyclin D1 immunoprecipitates of WI38 cell lysates and subsequently cloned (Xiong et al. 1993a). The *p21* gene encodes an arginine rich protein of 164 amino acids, with a cysteine rich region between amino acids 13 and 41

that could potentially bind zinc, and a very basic region between amino acids 140 and 163 with two potential nuclear localization signals (El-Deiry et al. 1993). In biochemical analyses, purified p21 assembles into tertiary complexes with multiple cyclin/CDKs, including cyclin D/CDK4, cyclin E/CDK2, cyclin A/CDK2 and cyclin B/CDC2. (Xiong et al. 1993a). Interestingly, the addition of increasing amounts of recombinant p21 to insect cell lysates containing various cyclins and CDK pairs led initially to increased formation of active complexes, although eventually resulted in the abrupt inhibition of kinase activity (Xiong et al. 1993a). This observation suggested that p21 could have a dual function, as an assembly factor when present at low concentrations, and as an inhibitor at high concentrations. Consistent with a CDK inhibitory function, p21 overexpression in mammalian cells blocked cell proliferation (Xiong et al. 1993a).

Four other research groups isolated p21 at approximately the same time using a variety of approaches. p21 was biochemically purified from CDK2 immunoprecipitates of mouse Balb/c 3T3 lysates (Gu et al. 1993). *p21* was also isolated in a yeast two hybrid screen designed to identify CDK2 binding proteins (Harper et al. 1993). In addition, *p21* was isolated in a expression screen designed to find genes involved in senescence (Noda et al. 1994). In this screen, an expression library from senescent cells was tested for its ability to block DNA replication of actively proliferating cells. This led to the identification of three inhibitors that induced cell cycle arrest when ectopically expressed or microinjected into cells (Noda et al. 1994). One of these genes was upregulated over 20 fold in senescent cells and was found to encode p21 (Noda et al. 1994). Interestingly, the upregulation of p21 in senescence was not simply a secondary event following quiescence, as p21 levels remained low in cells induced to enter quiescence by serum starvation (Noda et al. 1994).

Finally, *p21* was identified in a screen for transcriptional targets of the p53 tumor suppressor protein (El-Deiry et al. 1993). Using an inducible system to express p53 followed by a subtractive hybridization strategy, El-Deiry et al., isolated 28 cDNAs

corresponding to mRNAs that were highly upregulated following induction of wild-type p53 but not mutant p53 (El-Deiry et al. 1993). Significantly, all these cDNAs were derived from a single mRNA which encoded p21. Promoter analysis of the *p21* gene showed two consensus p53 binding sites (El-Deiry et al. 1992), which were each sufficient to provide p53 responsiveness to a heterologous gene expressed from a minimal promoter (El-Deiry et al. 1993). This suggested that *p21* was a direct transcriptional target of p53, and could perhaps be involved in the tumor suppressive function of p53. To test this idea, p21 was assayed for its ability to block tumor cell proliferation in a colony formation assay. The expression of *p21* in tumor cells resulted in a significant decrease in colony formation, suggesting that p21 was effective in growth suppression (El-Deiry et al. 1993). These data suggest that p21 may be an important target of p53-mediated tumor suppression.

The discovery of p21 by multiple groups using a variety of approaches provided a significant amount of information regarding p21 function. p21 was shown to have CDK inhibitory activity (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993a) and to be able to stop proliferation when ectopically overexpressed (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993a; Noda et al. 1994). In addition, p21 was upregulated in response to the tumor suppressor p53 (El-Deiry et al. 1993) and in cells which had become senescent (Noda et al. 1994).

Genomic structure

The human *p21* gene is composed of three exons of 68, 450 and 1600 bp that span a 10 kb region in the middle of the short arm of chromosome 6 (6 p21.2) (El-Deiry et al. 1993). The first ATG is located in the second exon at nucleotide 76, and an in-frame termination codon is present at nucleotide 570 (El-Deiry et al. 1993); hence, the majority

of the *p21* coding sequence is in exon 2. The p21 gene codes for a 2.1 kb transcript with a very long 3' UTR (~ two thirds of the mRNA). This message gives rise to a protein of 164 amino acids with an apparent molecular weight of 21 kDa in SDS-polyacrylamide gels. The human p21 protein is highly conserved and is 75% identical in primary amino acid sequence to the mouse p21 protein; sequence identity is particularly high in two regions, between amino acids 21-60 (95% identity) and amino acids 130-164 (89% identity) (Huppi et al. 1994). The mouse p21 gene is located on chromosome 17, in a region syntenic to human chromosome 6, and contains 3 exons with flanking introns whose position is conserved between the mouse and human genes (Huppi et al. 1994).

Mechanisms of inhibition of cell proliferation by p21

The p21 protein was originally discovered as part of a complex which contained a cyclin, a CDK, and PCNA (Xiong et al. 1992). This observation suggested that p21 could be important in the regulation of CDKs and PCNA. Furthermore, the inhibition of either CDKs or PCNA could explain the antiproliferative effects of p21. *p21* overexpression leads to a cell cycle arrest in the G1-phase of the cell cycle which could be mediated either through the inhibition of the G1/S CDKs, (cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2) or alternatively, through the inhibition of PCNA, a processivity factor for the principal replicative DNA polymerase, polymerase-d. The interaction between p21 and PCNA may be particularly important, as PCNA is the major protein associated with p21 in anti-p21 immunoprecipitates from IMR 90 human fibroblasts (Li et al. 1994b). Further insight was provided by an experiment in which p21 was added to an *in vitro* reconstituted SV40 DNA replication system, which was dependent on PCNA. The addition of p21 to this system blocked DNA replication at the elongation step but did not interfere with initiation of DNA replication (Flores-Rozas et al. 1994; Waga et al. 1994). These data

were consistent with an inhibitory effect of p21 on PCNA, and indeed, this inhibition could be overcome by the addition of purified PCNA (Flores-Rozas et al. 1994; Waga et al. 1994). These findings indicated that the inhibitory effects of p21 on cell proliferation could be due to inhibition of DNA replication.

PCNA is also essential for nucleotide excision repair, an important part of the cellular response to DNA damage induced by ultraviolet (uv) irradiation. In this process, short gaps of single stranded DNA are replicated in a PCNA-dependent manner following removal of damaged nucleotides. In yeast, this repair function of PCNA can be genetically separated from its role in DNA replication, suggesting that different activities are required for these two processes. The ability of p21 to inhibit the repair activity of PCNA was tested in an *in vitro* DNA repair system. It was found that p21 did not block the synthesis of short segments of DNA (Li et al. 1994a), thus, p21 seems to be a specific inhibitor of PCNA-dependent DNA replication.

Truncated forms of the p21 protein were used to map the minimal domain sufficient for the inhibitory effects of p21 on PCNA-mediated DNA replication. This domain was located in the C-terminus (Chen et al. 1995; Luo et al. 1995) and required amino acids 144-160 (Warbrick et al. 1995), which are included in one of the regions that is highly conserved between human and mouse p21 (Huppi et al. 1994). In addition, it was demonstrated that p21 directly bound PCNA *in vitro* in the absence of cyclins and CDKs (Flores-Rozas et al. 1994; Waga et al. 1994). This interaction was further characterized by solving the crystal structure of a C-terminal p21 peptide (amino acids 141-160) bound to PCNA (Gulbis et al. 1996). PCNA crystallized as a homotrimeric circular ring with a central hole wide enough to encircle double stranded DNA, which is consistent with its function as a clamp that tethers DNA polymerases to the sites of DNA replication. The p21 peptide bound PCNA with a 1:1 stoichiometry with an extended conformation across the outer face of each PCNA subunit, but leaving the central hole intact. p21

binding had minimal effects on PCNA structure, suggesting that the inhibitory effects of p21 on PCNA-dependent DNA replication were due to steric hindrance with other components of the DNA replication machinery. Consistent with this, an antibody directed against an epitope overlapping the p21 binding site on PCNA was reported to block DNA replication *in vitro*. Furthermore, it has been shown that the binding of p21 to PCNA can block PCNA binding to other proteins, such as cytosine-5 methyltransferase (Chuang et al. 1997) and Fen 1 (Chen et al. 1996b), an exonuclease which is necessary for the degradation of RNA-primer DNA junctions of immature Okazaki fragments.

The comparison between the quaternary structures of the C-terminus of p21 bound to PCNA and the conserved CKI inhibitory N-terminal domain of p27 bound to cyclin A/CDK2 supports the idea that p21 could simultaneously bind to both PCNA and cyclin/CDK complexes (Gulbis et al. 1996). Moreover, p21 has been shown to be required for the assembly of quaternary complexes containing cyclin, CDKs, p21 and PCNA (Xiong et al. 1993a). The significance of these complexes and the dual interaction of p21 with cyclin/CDKs and PCNA is not clear. However, the interaction of CKIs with PCNA is not without precedent, as this interaction has previously been documented for the Xenopus p27 ^{XIK1} (Su et al. 1995) and p28 ^{KIX1} (Shou and Dunphy 1996) inhibitors.

The significance of the inhibitory effects of p21 on PCNA-dependent DNA replication *in vivo* is unclear. In a *Xenopus* DNA replication system the addition of p21 was sufficient to block DNA synthesis (Strausfeld et al. 1994). However, unlike the inhibition observed in the SV40 DNA replication system, this inhibition could not be overcome by the addition of PCNA; in contrast, it was overcome by the addition of purified cyclin E (Strausfeld et al. 1994). These data strongly suggested that the inhibitory effects of p21 on DNA replication in this system were independent of PCNA. Furthermore, the effects of full length p21 protein could be recapitulated using a C-terminal truncated version of the protein which lacked the PCNA binding domain (Chen et al.

1995). Although DNA replication could also be inhibited by an N-terminal truncated form of p21, which contained the PCNA binding site, this inhibition was very inefficient and required protein concentrations 100-fold higher than those required for the C-terminal truncated form (Chen et al. 1995). Interestingly, the differences in the amounts of C-terminal and N-terminal truncated forms of p21 required for inhibition of DNA replication paralleled differences in concentration between the p21 binding-targets, CDK2 (2 mg/ml) and PCNA (200 mg/ml), in the *Xenopus* extract system (Shivji et al. 1994; Chen et al. 1995). These data illustrate the importance of the relative concentrations of p21 targets for a p21 activity. Given the abundance of PCNA relative to CDKs in proliferating cells, it is unclear whether p21 inhibits PCNA *in vivo*. Indeed, the amounts of an N-terminal truncated form of p21 required to inhibit proliferation in transient transfection assays were reported to be significantly higher than those required for a C-terminal truncated protein (Luo et al. 1995). Furthermore, the subcellular localization patterns of p21 and PCNA seems to have minimal overlap in normally proliferating cells (Li et al. 1996). It is possible that p21 does regulate PCNA *in vivo*, but only under specific circumstances. For instance, after uv irradiation, p21 and PCNA are both upregulated and colocalize in the nucleus, where p21 could potentially inhibit PCNA function (Li et al. 1996).

p21 can also function as an effective inhibitor of G1/S CDKs *in vitro* (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993a). In contrast to other members of the CIP/KIP family, p21 contains two motifs that can interact with cyclins: Cy1 (amino acids 17-24) which is in the N-terminus, and Cy2 (amino acids 152-158) which is in the C-terminus and overlaps with the PCNA binding site (Chen et al. 1996a). At least one of these motifs, together with the CDK binding site, is required for efficient cyclin/CDK inhibition by p21 (Chen et al. 1996a). The ability of p21 to inhibit cyclin/CDK complexes has been examined using purified components (Harper et al. 1995). Recombinant p21 efficiently inhibits cyclin A/CDK2 and cyclin D/CDK4 complexes preassembled in insect

cells with a K_i ~0.5 nM. Cyclin E/CDK2 inhibition required slightly higher concentrations of p21 (K_i ~3.7 nM). p21 also inhibited cyclin B/CDC2, albeit with a significantly lower efficiency (K_i ~400 nM). These data indicate that p21, like the other CIP/KIP proteins, has a broad range of cyclin/CDK targets. The interaction of p21 with multiple cyclin/CDK complexes may involve conserved structural motifs, or may reflect an ability of p21 to adopt multiple conformations (Kriwacki et al. 1996). Interestingly, it has been found that p21 lacks a stable secondary and tertiary structure in the free solution state but adopts a rigid conformation upon binding to CDK2; thus, p21 may be able to adopt multiple stable conformations upon binding to different cyclin/CDK complexes (Kriwacki et al. 1996). *In vitro,* p21 did not bind cyclin H/CDK7 (CAK) (Harper et al. 1995). However, p21 could inhibit phosphorylation of cyclin A/CDK2 by CAK (Aprelikova et al. 1995). This effect is likely to be due to steric interference caused by p21 binding to cyclin A/CDK2 and preventing CAK phosphorylation, as supported by crystallographic data (Russo et al. 1996a). In addition, p21 can sterically block cyclin A/CDK2 and cyclin E/CDK2 binding to CDC25A (Saha et al. 1997), which is necessary under some circumstances for kinase activation. Thus, p21 can inactivate cyclin/CDK complexes by multiple mechanisms.

It has been proposed that cyclin/CDK inhibition by p21 requires binding of multiple molecules of p21, but that at lower concentrations, p21 may act as a cyclin/CDK assembly factor (Zhang et al. 1994b; Zhang et al. 1994a; LaBaer et al. 1997). This model is supported by the observation that CDK2 complexes containing kinase activity can be recovered by immunoprecipitation with antibodies against p21 (Zhang et al. 1994b). Furthermore, in some fibroblast cell lines the majority of CDK2 is associated with p21, and immunodepletion of these complexes with a-p21 antibodies results in a significant reduction of the total CDK2 kinase activity (Harper et al. 1995). Also the addition of small amounts of purified p21 to lysates containing cyclins A or E and CDK2 results in enhanced complex formation and increased kinase activity (Xiong et al. 1993a). Cyclin

A/CDK2 complexes bound to p21 migrate at two different sizes in gel filtration chromatography, one of which coincides with the predicted size for a ternary p21/cyclin A/CDK2 complex and another which has a higher molecular weight (Zhang et al. 1994b). Analysis in parallel of the kinase activities of these complexes showed that while the small complexes were active, the large ones were inactive. To address whether the large complexes contained more than one subunit of p21, two differentially labeled p21 subunits were added to the extracts containing cyclin A and CDK2 (Zhang et al. 1994b). This experiment showed that the small complexes contained only one p21 subunit per complex, while the large complexes contained multiple p21 subunits (Zhang et al. 1994b). These data support the model that more than one molecule of p21 is required for kinase inhibition. However, this conclusion is in sharp contrast with predictions based on the crystal structure of the conserved CKI binding domain of p27 bound to cyclin A/CDK2, which showed that a single CKI domain was sufficient to inactivate the kinase (Russo et al. 1996b). In addition, the authors reported that stable complexes containing more than one N-terminal domain could not be generated even at high concentrations of p27 (Russo et al. 1996b).

p21 function

- **p53 tumor suppressor pathway**

p53 is a tumor suppressor gene that is mutated in over 50% of sporadic human tumors (Hollstein et al. 1991). Moreover, individuals who inherit one defective copy of the *p53* gene are highly predisposed to tumorigenesis (reviewed by Harris and Hollstein 1993). Data from knock-out mice has shown that this important tumor suppressor is not required for normal development (Donehower et al. 1992; Clarke et al. 1994; Jacks et al. 1994;

Purdie et al. 1994), but does play a critical role in the response of cells to genotoxic stress (Kastan et al. 1992; Clarke et al. 1993; Lowe et al. 1993; Clarke et al. 1994; Merritt et al. 1994). DNA damage results in the activation of p53, leading to a cell cycle arrest or apoptosis depending on the cell-type (reviewed by Levine 1997). For instance, fibroblasts undergo a p53-dependent cell cycle arrest in the G1 phase in response to irradiation (Kastan et al. 1991; Kastan et al. 1992). In contrast, immature, typically rapidly dividing cells, such as stem cells of the intestinal crypts or immature thymocytes, undergo p53-dependent apoptosis in response to irradiation (Clarke et al. 1993; Lowe et al. 1993; Merritt et al. 1994). These anti-proliferative activities of p53 are thought to be critical for preventing the accumulation of oncogenic mutations (reviewed by Lane 1993).

At the molecular level, p53 acts as a sequence specific transcriptional activator (reviewed by Ko and Prives 1996). The transcriptional activities of p53 are important for its growth suppressive function, as illustrated by the observation that the majority of p53 point mutations found in tumors result in amino acid changes in the DNA binding domain, which in turn is essential for transactivation (reviewed by Harris and Hollstein 1993). In addition, Attardi et al., has clearly shown that transcriptional activation by p53 is necessary for its growth suppressive properties (Attardi et al. 1996). In these studies, p53 mutants defective in transcriptional activation failed to induce growth arrest or apoptosis when microinjected into normal fibroblasts or fibroblasts primed to undergo cell death by the expression of the viral oncogene *E1A* (Attardi et al. 1996).

p21 is a p53 responsive gene, and may be a critical target of p53 in tumor suppression. Like p53, overexpression of *p21* in tumor cell lines inhibits proliferation (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993a). The *p21* promoter contains two p53 binding sites at 1.9 and 2.8 kb upstream of the transcription initiation site (El-Deiry et al. 1995). These regions are within two of the three segments of the *p21* promoter that are highly conserved between humans and mice, indicating that these sites are likely to be

important for p21 function (El-Deiry et al. 1995). p21 is a direct p53 transcriptional target and DNA damage results in the accumulation of both the p53 and p21 proteins (El-Deiry et al. 1994). *In vivo* footprinting studies have shown increased p53 binding to the p53 recognition sites in the *p21* promoter following irradiation (Chin et al. 1997). Significantly, p21 upregulation occurs both in cells undergoing p53-dependent arrest as well as in cells initiating p53-dependent apoptosis in response to DNA damage, suggesting that p21 may be implicated in both of these responses (El-Deiry et al. 1994).

The p21 protein may provide a mechanism to link p53 activation to cell cycle arrest. Ionizing-radiation of fibroblasts results in a cell cycle arrest in G1, which is accompanied by inhibition of the G1 CDKs, cyclin D/CDK4 (Terada et al. 1995) and cyclin E/CDK2 (Dulic et al. 1994). This arrest correlates with the accumulation of a cyclin E/CDK2 inhibitory activity that can be depleted with antibodies against p21 (Dulic et al. 1994). Importantly, this inhibitory activity is only generated in cells containing wild-type p53 (Dulic et al. 1994). Taken together, these data suggest that p21 may be responsible for the inhibition of cyclin E/CDK2 in response to p53 activation by DNA damage.

It is not clear how p21 participates in the process of p53-dependent apoptosis. Two opposite functions for p21 have been proposed. Specifically, p21 could be a mediator of apoptosis (El-Deiry et al. 1994) or, alternatively, it could protect against apoptosis (Canman et al. 1995; Polyak et al. 1996). In support of the second view, it has been shown that the inactivation of p21 by gene targeting in colon cancer cells switches their response to irradiation from G1 arrest to apoptosis (Polyak et al. 1996). According to this model, p21 upregulation in response to irradiation would be necessary to implement a G1 arrest and in the absence of p21, failure to arrest would result in programmed cell death. According to the other model, p21 would actively promote apoptosis.

- **p53-independent functions of p21**

Studies of p21 in cells lacking p53 indicated that p21 expression can be regulated independently of p53 in response to a variety of signals. Treatment of *p53-/-* MEFs with either serum or purified growth factors, such as PDGF, results in the upregulation of *p21* expression (Michieli et al. 1994). This upregulation occurs in the absence of protein synthesis, indicating that p21 belongs to the class of immediate early genes (Michieli et al. 1994). The significance of the upregulation of an inhibitor of cyclin-dependent kinases in response to proliferative signals is not clear. Nevertheless, this data clearly indicate that p21 is involved in processes other than the p53-mediated DNA damage response.

Additionally, p21 may play a role in differentiation. There are many assays that are thought to simulate the process of differentiation in tissue culture. For instance, many tumor cell lines can be induced to express differentiation markers and permanently withdraw from the cell cycle in response to different chemical agents. These treatments often result in an upregulation of p21 protein, which occurs independently of p53 status (Jiang et al. 1994; Steinman et al. 1994; Macleod et al. 1995; Zhang et al. 1995). These data suggest that p21 is important for differentiation in tissue culture model systems, and that this role is independent of p53.

The analysis of the *p21* promoter has revealed recognition sequences for several transcription factors such as AP2 (Zeng et al. 1997), vitamin D3 receptor (Liu et al. 1996b), retinoic acid receptor (Liu et al. 1996a), C/EBPa (Timchenko et al. 1996), and MyoD (Halevy et al. 1995), all of which regulate gene expression during differentiation. Furthermore, all of these transcription factors have been shown to bind to the recognition sequences in the p21 promoter in electro-mobility shift assays (EMSA) (170-174). In addition, when fused to a reporter gene with a minimal promoter, these sequences conferred reporter inducibility in response to ectopic expression of the transcriptional activators (170-174). These data indicate that p21 may act in multiple differentiation

pathways.

Analysis of *p21* expression in mice and humans supports the idea that p21 is important for differentiation *in vivo*. In mouse embryos, the pattern of expression of *p21* analyzed by *in situ* hybridization correlated with terminal differentiation in multiple cell lineages, including skeletal muscle, skin, respiratory epithelium and cartilage (Parker et al. 1995). In addition, in the adult mouse, the highest levels of *p21* mRNA were detected in the intestine (Macleod et al. 1995), an organ that contains a vast number of cells undergoing terminal differentiation due to a large epithelial surface that has a fast turn over. Immunohistochemistry studies performed in humans confirmed that the expression of p21 in the intestine is confined to the postmitotic, fully differentiated compartment (El-Deiry et al. 1995). With the exception of the spleen, *p21* expression in the embryo and the adult mouse appears to be independent of p53, as the expression pattern of *p21* remains unchanged in *p53*-deficient embryos and adult mice (Macleod et al. 1995; Parker et al. 1995).

Other data suggest that p21 is involved in the cell cycle withdrawal associated with terminal differentiation. Muscle differentiation is regulated by a family of basic-HLH transcription factors including MyoD (Lassar et al. 1994). In fibroblasts, ectopic expression of MyoD under conditions of low serum induces the expression of muscle specific genes and the formation of myotubes (Lassar et al. 1994). As part of this process, cells undergo an irreversible withdrawal from the cell cycle, which occurs in G1 and is associated with the upregulation of p21 (Halevy et al. 1995) and the inhibition of the G1 CDKs, CDK4 and CDK2 (Guo et al. 1995). This differentiation-associated arrest may only require p21, as in a transgenic model system, *p21* overexpression was shown to be sufficient to irreversibly block proliferation (Wu et al. 1996).

The importance of p21 in the cell cycle arrest associated with terminal differentiation is underscored by the observation that p21 is a target of a papilloma virus

oncoprotein (Funk et al. 1997; Jones et al. 1997). Papilloma viruses are DNA viruses that infect differentiated epithelial cells and cause benign hyperplasias or warts. These viruses must stimulate their host cells to proliferate in order to replicate productively. Therefore, papilloma viruses have developed strategies to subvert the arrest machinery of differentiated epithelial cells. Human papilloma virus 16 (HPV16) encodes one such protein, the E7 oncoprotein, which inactivates pRB, an important inhibitor of cell proliferation. Recently it has been reported that the E7 protein also binds to and inactivates p21 (Funk et al. 1997; Jones et al. 1997). E7 binds to p21 both *in vivo* and *in vitro*, and blocks the inhibitory effects of p21 on cyclin E/CDK2 (Funk et al. 1997; Jones et al. 1997) and PCNA-mediated DNA replication (Funk et al. 1997). These data support the idea that p21 is an important inhibitor of cell proliferation in differentiated cells *in vivo*.

In addition to implementing a cell cycle block, p21 may be important for other activities in differentiated cells. For example, p21 has been shown to protect differentiating cells from entering apoptosis. The myoblastic cell line C2C12 can be induced to differentiate by reducing the concentration of growth factors present in the media. Under these conditions, formation of myotubes occurs but is accompanied by cell death of a significant fraction of the cell population. Interestingly, the majority of cells undergoing apoptosis were seen to express low levels of *p21* (Wang and Walsh 1996), leading to the hypothesis that p21 may protect differentiating cells from apoptosis. To test this model, C2C12 cells were transfected with p21 and then induced to differentiate by serum deprivation. Overexpression of full-length *p21*, but not of a truncated form lacking the CKI domain, was seen to protect cells from apoptosis (Wang and Walsh 1996). A role for p21 in protecting cells from apoptosis is not limited to this particular cell line. Reducing the expression of p21 with antisense oligonucleotides in neuroblastoma cells induced to differentiate in tissue culture resulted in high levels of cell death (Poluha et al. 1996). These results suggest that expression of p21 in differentiating cells may protect them from cell

death.

The findings discussed above imply that p21 is an important mediator of cell cycle arrest in many cell types induced to differentiate. In addition, p21 may be critical to protect cells from death during the process of differentiation.

To determine the role of p21 in differentiation as well as in p53-mediated growth suppression, we have generated two different mouse models, a chimeric mouse composed partly of wild-type and partly of p21-deficient cells and a p21-deficient mouse. These models have been extremely useful to understand the function of p21 *in vivo* and will be described in the following chapters.

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Chapter 2

Radiation-induced Cell Cycle Arrest Compromised by p21 deficiency

The work presented in this chapter is taken largely from a paper publised in *Nature* (Brugarolas et al. 1995). My contributions to this chapter include the following:

Generation of *p21+/-* as well as *p21-/-* ES cells.

The generation of chimeric mice.

The derivation of pure populations of *p21*-deficient MEFs from chimeric embryos.

The analysis of the irradiation response of *p21*-deficient MEFs.

The generation of a *p21*-deficient mouse strain.

In addition this chapter includes data generated by G. J. Hannon:

Construction of the p21 targeting vector.

Screen of ES cells for homologous recombination events at the *p21* locus.

Characterization of cylcin-CDK complexes as well as the p53 status in *p21-/-* MEFs.

In vitro CDK2 kinase assays of untreated and irradiated *p21*-deficient cells.

C. Chandrasekaran has alco made contributions to this chapter, in particular, the histological analysis of the intestinal epithelium of the chimeric mice.

ABSTRACT

p21 is a dual inhibitor of cyclin dependent kinases[1-3] and proliferating-cell nuclear antigen (PCNA)[4], both of which are required for passage through the cell cycle. The *p21* gene is under the transcriptional control of p53[5], suggesting that p21 might promote p53-dependent cell cycle arrest or apoptosis. p21 has also been implicated in cell senescence[6] and in cell cycle withdrawal upon terminal differentiation[7-9]. Here we investigate the role of p21 in these processes using chimeric mice composed partly of *p21-/-* and partly of *p21+/+* cells. Immunohistochemical studies of the *p21+/+* and *p21-/-* components of adult small intestine indicated that deletion of *p21* had no detectable effect on the migration-associated differentiation of the 4 principal intestinal epithelial cell lineages or on p53-dependent apoptosis following irradiation. However, *p21-/-* mouse embryo fibroblasts are impaired in the ability to undergo G1 arrest following DNA damage.

RESULTS

To investigate the many proposed functions of p21, a null allele was generated in 129/Sv embryonic stem (ES) cells by replacing the *p21* coding sequence with a neomycin resistance cassette (neo^r; see Figure 1A-C). Homozygous *p21-/-* ES cells were produced by subjecting two heterozygous *p21+/-* cell lines to selection in an elevated concentration of G418. One *p21-/-* ES cell clone was recovered from each parental *p21+/-* line. *p21*-deficiency was verified by Southern blot analysis (Fig. 1B, C) and by immunoprecipitation of ES cell lysates with a p21 polyclonal antiserum (data not shown).

The role of p21 in intestinal epithelial differentiation

The small intestine was chosen as a model for assessing the role of p21 in proliferation, differentiation and cell death[10]. Studies of adult aggregation chimeras indicate that each intestinal crypt is monoclonal whereas each villus receives cells from 2-3 surrounding crypts. Introduction of 129/Sv ES cells, such as those lacking *p21*, into normal B6 blastocysts results in B6↔129/Sv chimeras that contain patches of B6 crypt-villus units and patches of 129/Sv crypt-villus units. These can be readily distinguished based on differences in their ability to bind the lectin, UEA-1[11]. In B6↔129/Sv chimeras, proliferation was restricted to undifferentiated epithelial cells in both *p21+/+* and *p21-/-* crypts (Fig. 2A-C). Furthermore, multilabel immunohistochemical surveys with a well characterized panel of antibodies and lectins[11] revealed that loss of *p21* had no detectable effects on the ability of the intestine to complete normal morphogenesis or to maintain the differentiation programs of its 4 principal epithelial cell lineages (e.g. see Figure 2F).

p21 is not necessary for p53-mediated apoptosis

Low to moderate doses of γ-irradiation cause cell cycle arrest and p53-dependent apoptosis in crypts[12-14]. Irradiation caused the same 100-150 fold increase in the frequency of cell death in crypts composed either of *p21*+/+ or of *p21*-/- cells (Fig. 2G,H), indicating that p21 is not required for p53-dependent cell death, at least in this system. *p21*-/- and *p21*+/+ crypts also showed a similar reduction in 5-bromodeoxiuridine (BrdU)- positive cells following irradiation. Thus, radiation-induced cell cycle arrest occurred in undifferentiated intestinal epithelial cells regardless of p21 status (Fig. 2D,E). However, as cell cycle arrest in these cells has not been shown to require p53, therefore we examined the role of p21 in DNA damage-induced G1 arrest in fibroblasts, which is p53-dependent.

Derivation of *p21*-deficient fibroblasts from chimeric embryos

Cultures of primary mouse embryo fibroblasts (MEFs) were prepared from B6↔129/Sv p21-/- chimeras produced from two different *p21*-/- ES cell lines (p21 A and p21 B) and from B6↔129/Sv *p53*-/- chimeras. MEFs were also prepared from wild-type embryos and from chimeras generated from an ES cell line carrying a random integration of the *p21* targeting vector. Initial MEF cultures were 30-70% 129/Sv (Fig. 3A). Highly pure MEF populations (>95% 129/Sv) were obtained following G418 selection (Fig. 3A). It should be noted that this enrichment required no more than two population doublings. The absence of p21 protein in the *p21*-/- MEFs was confirmed by immunoprecipitation (Fig. 3B,C), and the purity of *p21*-deficient cell populations was verified by immunofluorescence with a p21 specific antiserum (data now shown).

Immunoprecipitation with anti-CDC2,-CDK2 or -CDK6 antibodies showed that the pattern of cyclin-CDK complexes was indistinguishable in the *p21*-/- and *p53*-/- MEFs, although the *p21*-/- MEFs still contain wild-type p53 as judged by immunoprecipitation

with conformation-specific p53 antibodies (Fig. 3D). In these cells, p21 was absent from CDK2 and from CDC2 complexes, and CDK6 was primarily associated with p16 (Fig. 3C). In contrast to *p53-/-* MEFs, *p21-/-* cell populations did not show obvious differences in proliferation rates compared to normal controls (not shown).

p21 is an important p53 target in G1 arrest

The two *p21-/-* MEF populations along with *p53-/-*, random integration and wild-type controls were treated with γ-radiation and subjected to cell cycle analysis. The G1 radiation response was assessed by comparing the S-phase fraction in irradiated cells (18 hours after a dose of 5.5 Gy) to that in untreated cell populations. Wild-type cells and cells carrying the random integration were substantially arrested in G1 following irradiation (Fig. 4A). The fraction of cells in S-phase after irradiation was 39.5% or 39%, respectively, of that in non-irradiated cells. For the *p53-/-* MEFs, the S-phase fraction after irradiation was 84.5% of the value in untreated cells, a result that is consistent with previous reports demonstrating that p53 is required for G1 cell cycle arrest following DNA damage in fibroblasts[15]. The MEF populations derived from the two *p21-/-* ES cell clones showed an intermediate irradiation response, with post-irradiation S-phase fractions at 57.5% or 54.5% of the level in non-irradiated samples. Similar results were obtained in six independent experiments (Fig. 4A,B).

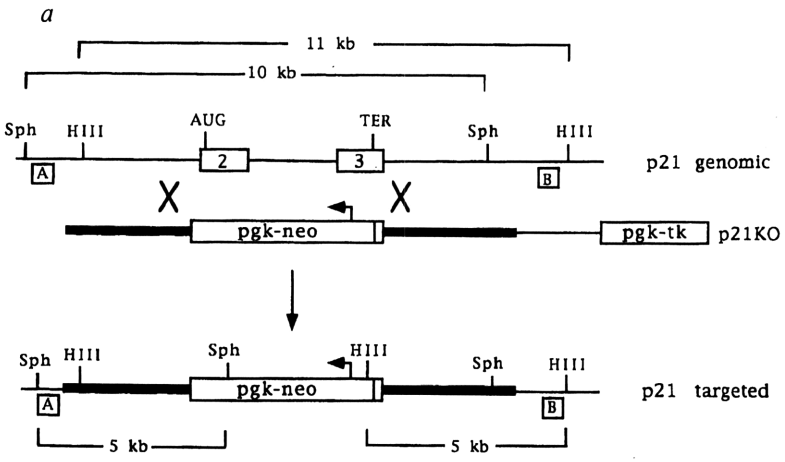
Several models could explain the intermediate phenotype of *p21-/-* MEFs. For example, *p21-/-* cells could differ from wild type cells in the timing of the radiation response or in the amount of damage required to induce arrest. We therefore examined the radiation response of *p21-/-* MEFs at several times following treatment (14, 18 and 22 hours after a dose of 5.5 Gy) and at two different radiation doses (5.5 Gy and 11 Gy, analyzed at 18 hours post irradiation). In each case, our results were similar to those

shown (Fig. 4A,B). The intermediate phenotype of *p21-/-* cells could alternatively reflect the fact that primary MEF are composed of a mixture of cell types, each of which may depend to a different extent on p21 for growth arrest in the presence of DNA damage. Finally, the position of cells in the G1 phase could determine sensitivity to the p21-dependent mechanism of p53-induced cell cycle arrest.

It is well established that, following irradiation, the activity of CDK2 kinase is reduced by approximately 3-5 fold in human fibroblasts[16]. The same is true in normal MEFs (Fig 4C, ~5-fold reduction). However, inhibition of CDK2 is not observed upon irradiation of *p53-/-* or *p21-/-* cells (Fig 4C). This demonstrates that DNA damage-induced inhibition of CDK2 requires p21.

Figure 1. *p21* gene targeting in embryonic stem (ES) cells.

a, Targeting scheme. The targeting vector, p21KO, contains approximately 8 kb of *p21* genomic sequences (denoted by thick lines) flanking the pgk-neo expression cassette which is located in the opposite transcriptional orientation to *p21*. Recombination on the 5' side of *p21* was monitored by Southern blotting using an *SphI* digest and probe A. The 3' recombination junction was characterized with a *HindIII* digest of genomic DNA and probe B. Both probes lie outside the genomic regions present in the targeting vector. ***b*** and ***c***, Southern blot analysis of ES cell clones. DNA from the parental D3 ES cells, two heterozygous mutant clones (*p21+/-* A and B) and two homozygous mutant clones that were derived from the heterozygous clones following exposure to increased G418 concentrations (*p21-/-* A and B) are shown. Using probe A on *SphI* digested genomic DNA (panel B), the *p21+/-* clones show a ~5 kb mutant-specific band (band d) in addition to the ~10 kb band (band w) corresponding to the wild-type *p21* allele. In the *p21-/-* cell lines, the wild-type band is absent. At the 3' recombination junction (*HindIII* digested DNA and probe B, panel C), *p21+/-* A shows the expected mutant specific band of ~5 kb (band d), while the mutant band in clone B is approximately 3.6 kb (band d*). Extensive restriction mapping revealed that p21+/-B contained a 1.4 kb deletion 3' to exon 3 of *p21*. In both *p21-/-* lines, the band corresponding to the wild-type allele of *p21* is again absent.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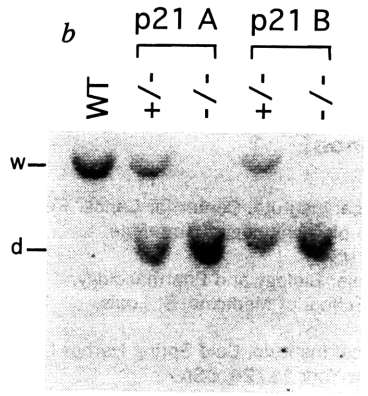
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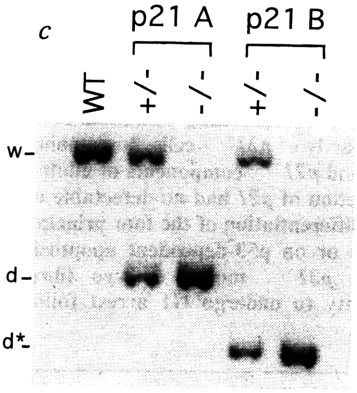
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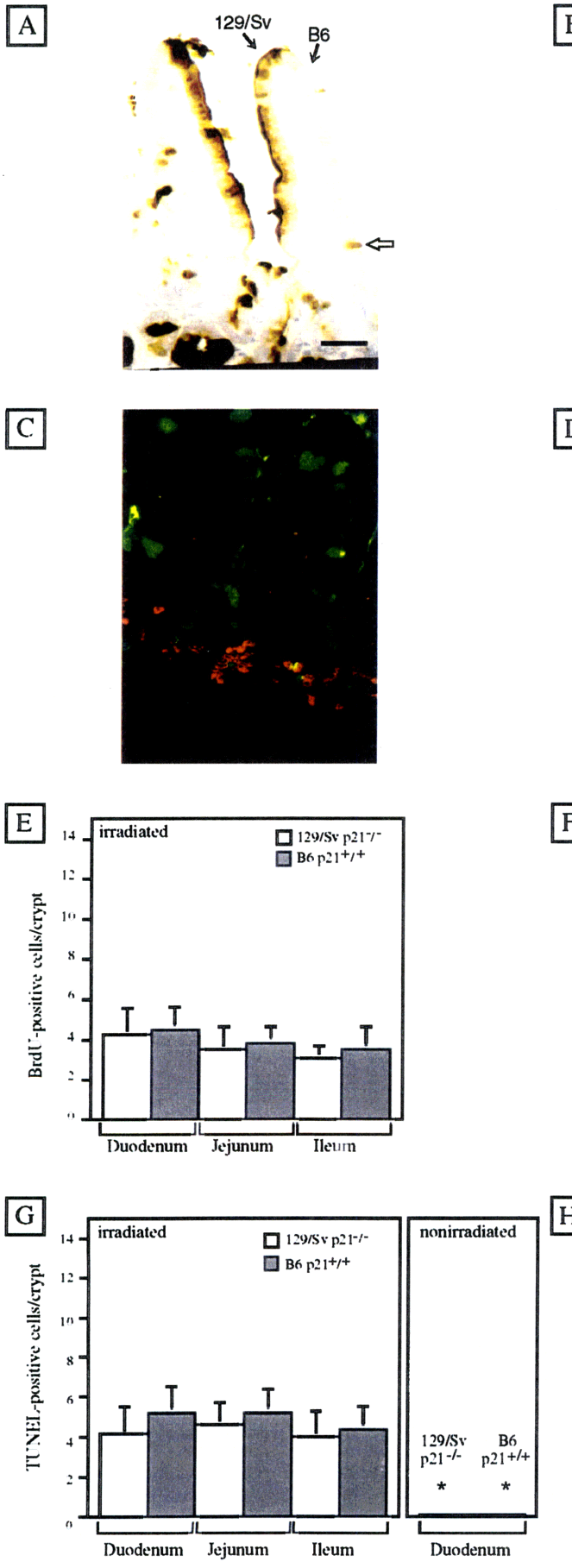
Figure 2. Use of B6↔129/Sv *p21-/-* chimeric mice to assess the role of p21 in proliferation, differentiation and death programs.

A, A polyclonal jejunal villus from a chimeric mouse stained with peroxidase-conjugated UEA-1 is shown. 129/Sv but not B6 enterocytes bind the lectin . A subset of B6 and 129/Sv goblet cells are also labeled (open arrow). **B, C** Adjacent patches of *p21+/+*B6 and *p21-/-*129/Sv jejunum (panels B and C respectively) were stained with FITC-conjugated UEA-1 (green) and anti-BrdU (red). **D, E** S-phase cells were quantitated in crypts from irradiated (panel E) and non-irradiated (panel D) chimeras following staining with UEA-1 and anti-BrdU. 30 crypts were counted/section/region/mouse (n=3 mice each). Mean values ± 1 S.D. are plotted. **F**, Adjacent B6 and 129/Sv villi show similar patterns of expression of liver fatty acid binding protein (L-FABP), a differentiation marker for the enterocytic lineage. The section was stained with FITC-UEA-1 (green) and rabbit anti-L-FABP sera (red). **G, H**, A quantitation of p53-dependent apoptosis in crypts from irradiated animals is shown (TUNEL assay). The asterisk denotes that non-irradiated age matched B6↔129Sv *p21-/-* chimeras contain on average only one TUNEL-positive cell per 30 crypts. Bar (in panel A) = 25 μm.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129/Sv
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129/Sv p21-/-
B6 p21+/+
BrdU-positive cells/crypt
Duodenum
Jejunum
Ileum
E
irradiated
129/Sv p21-/-
B6 p21+/+
BrdU-positive cells/crypt
Duodenum
Jejunum
Ileum
F
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G
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nonirradiated
129/Sv p21-/-
B6 p21+/+
TUNEL-positive cells/crypt
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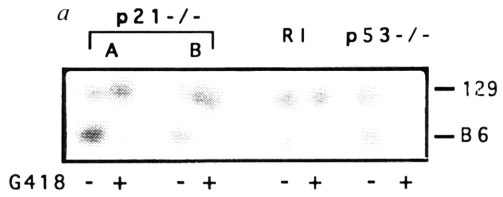
Ileum

Figure 3. Selection of targeted mouse embryo fibroblasts (MEFs).

a, Glucose-6-phosphate isomerase (GPI) isoenzyme analysis. Donor ES cells (strain 129/Sv) contain a GPI isoenzyme with a different electrophoretic mobility than that of the recipient C57BL/6 blastocysts. The ratio of these isoforms was used to estimate the contribution of the targeted and recipient cells to MEF populations. GPI analysis is shown for the initial MEF preparations from representative chimeras generated from each ES cell clone (as indicated; RI, random integration) and for MEFs enriched for cells derived from targeted ES cells by selection with G418. ***b***, *p21-/-* MEFs lack p21 protein. MEF populations (as indicated) were tested for the presence of p21 by immunoprecipitation. Note that p21 and associated proteins are present in the unselected *p21-/-* populations but are barely detectable following selection. ***c***, Cyclin-CDK complexes in *p21-/-* cells. Cyclin-CDK complexes were recovered from the indicated cell populations using antisera against p21, CDC2, CDK2 and CDK6. The identities of co-precipitated proteins are indicated. ***d***, Immunoprecipitation of p53 with conformation specific antibodies. p53 protein was recovered from lysates of ^{35}S-labeled cells (indicated) by immunoprecipitation with antibodies specific for either the wild-type (wt, PAb 1620, Oncogene Sciences) or the mutant (mut, PAb 240, Oncogene Sciences) conformations of p53.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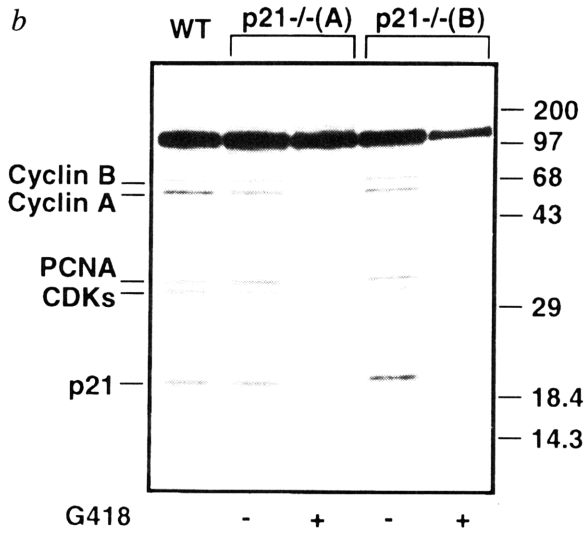
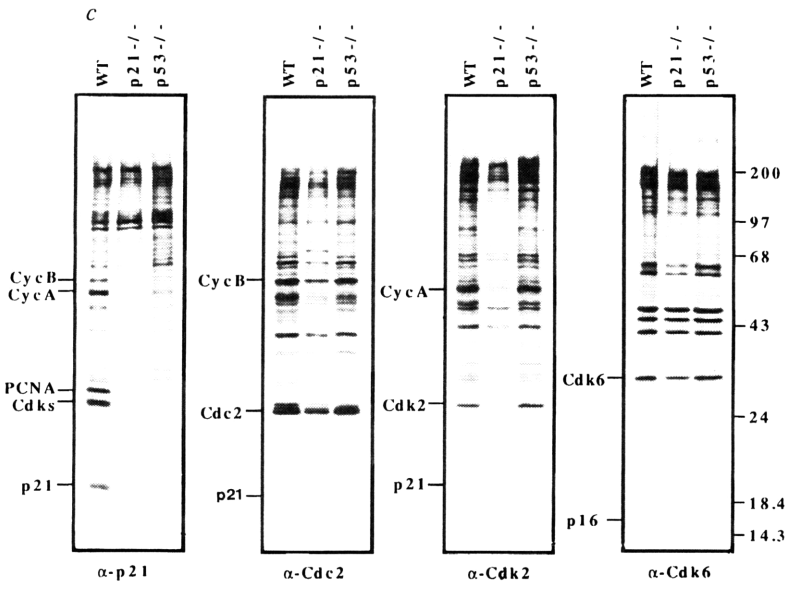
A
B
RI
p53-/-
129
B6
G418 - + - + - + - +


b
WT
p21-/-(A)
p21-/-(B)
200
97
68
43
29
18.4
14.3
Cyclin B
Cyclin A
PCNA
CDKs
p21
G418 - + - +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p21-/-
p53-/-
CycB
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PCNA
Cdks
p21
α-p21
Cdc2
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97
68
43
24
18.4
14.3

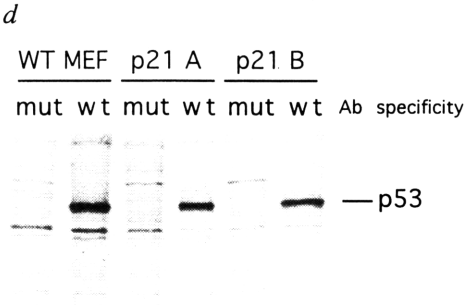
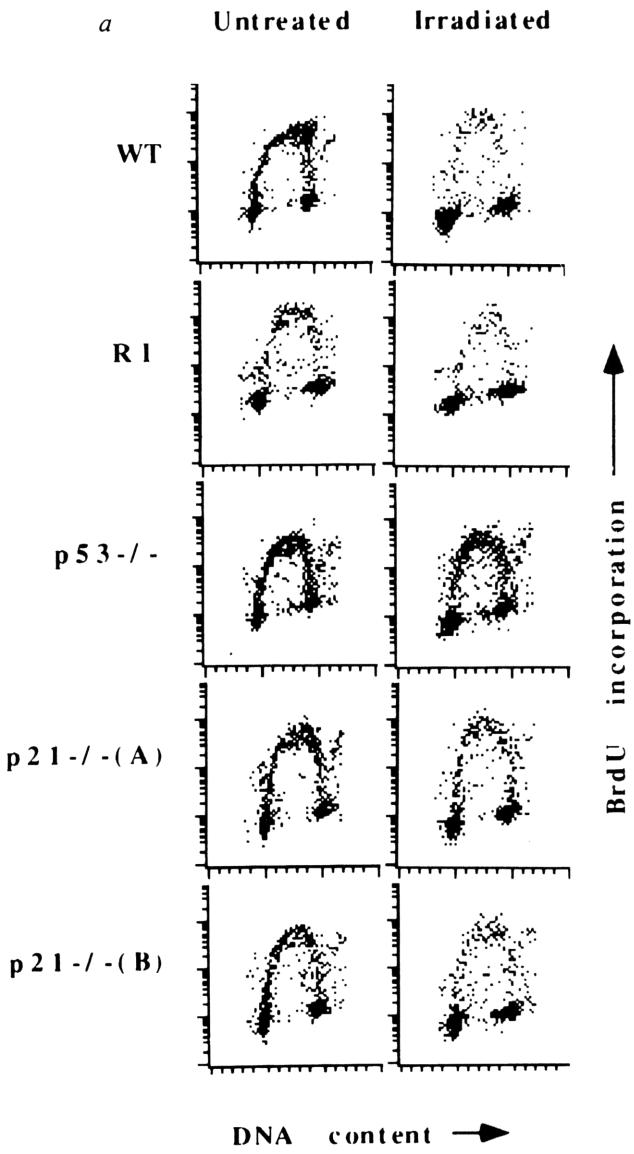

d
WT MEF
p21 A
p21 B
mut wt mut wt mut wt Ab specificity
p53

Figure 4. Radiation response of *p21-/-* cells.

a, Representative two-dimensional FACS analysis of untreated and irradiated samples of *p21-/-*, *p53-/-*, random integration (RI) and wild-type (WT) MEFs are shown. Asynchronous cultures were irradiated with a dose of 5.5 Gy and the percentage of cells synthesizing DNA was evaluated with a 4 hour BrdU pulse, beginning 14 hours after irradiation. Cells in the G0 or G1 phase of the cell cycle appear in the lower left quadrant. Cells in the G2 or M phases appear in the lower right quadrant. S-phase cells are positive for BrdU and are detected in the arch that connects G0/G1 and G2/M populations. ***b***, Histogram showing the S-phase fraction in irradiated versus untreated samples. A quantitation of the number of cells remaining in S-phase following irradiation compared to the number of S-phase cells in an untreated control is shown. The mean of 6 independent experiments is shown for all samples except the random integration (3 independent experiments). Error bars show standard deviations. ***c***, Activity of CDK2 kinase following irradiation. CDK2 immunoprecipitates from either irradiated or non-irradiated MEF populations (as indicated) were prepared at 18 hours following treatment with 5.5 Gy. Precipitates were tested for the ability to phosphorylate histone H1.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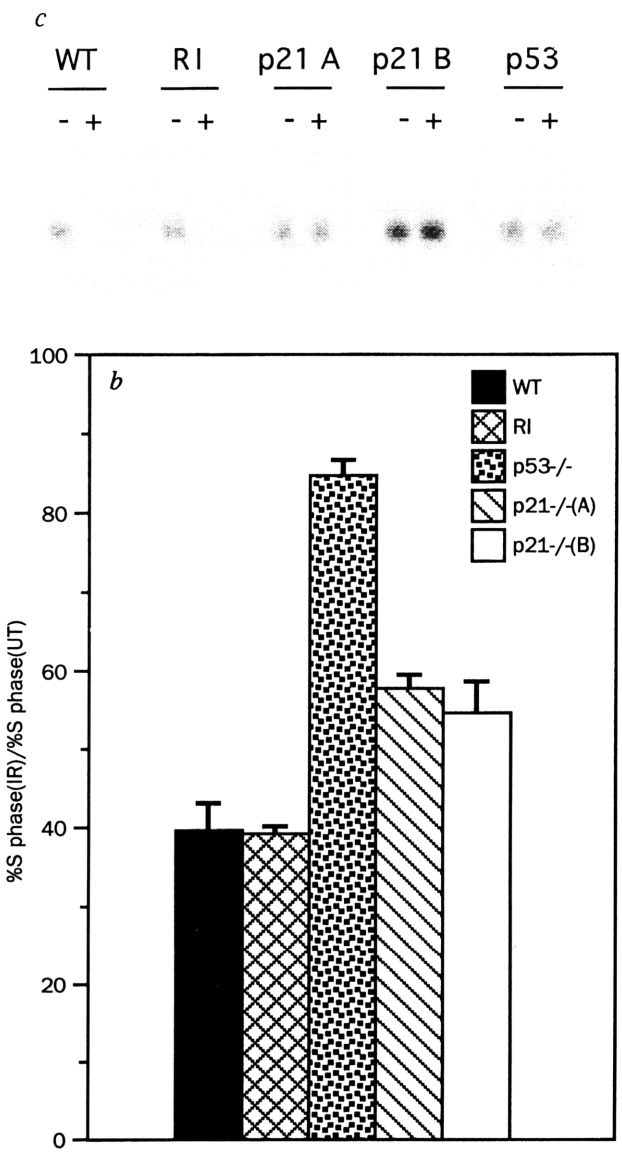
Untreated
Irradiated
WT
R1
p53-/-
p21-/-(A)
p21-/-(B)
BrdU incorporation
DNA content


c
WT
R1
p21 A
p21 B
p53
- +
b
WT
RI
p53-/-
p21-/-(A)
p21-/-(B)
%S phase(IR)/%S phase(UT)
100
80
60
40
20
0

DISCUSSION

Since its discovery[17], p21 has been implicated in a broad spectrum of biological processes. Inhibition of cell proliferation by p21 has been linked to the permanent cell cycle withdrawal that accompanies senescence.[6] As a transcriptional target of p53, p21 has been proposed as an effector of cell cycle arrest following DNA damage, as a component of the cell death program and as a tumor suppressor. Our data indicate that p21 is not required for p53-dependent apoptosis (at least in some cell types). However, the consequences of *p21* disruption clearly demonstrate the involvement of this protein in p53-dependent cell cycle arrest following DNA damage. Our data also imply the existence of additional p53-responsive growth control mechanisms. This may help to explain the lack of *p21* mutations in human tumors[18]. We have followed a small number of chimeric *p21-/-* mice (up to 80% *p21-/-* as judged by coat color) for more than one year. The fact that none of these animals appears predisposed to cancer provides further evidence against a role for p21 as a major tumor suppressor. Several studies have also suggested a role for p21 during differentiation in a number of cell lineages[7-9]. Our data demonstrate that p21 is not essential for terminal differentiation in the small intestine. Furthermore, we have generated a germline, homozygous *p21*-deficient animal. The *p21-/-* mouse is viable and displays no apparent phenotype by 7 weeks of age. Thus, the fact that p21 is not solely responsible for p53-dependent growth arrest or cell cycle withdrawal following differentiation could indicate a general need for tight regulation of cell proliferation by several overlapping pathways.

EXPERIMENTAL PROCEDURES

Gene targeting

Gene targeting was performed using D3 ES cells as described[19-21]. *p21-/-* ES cell clones were generated from *p21+/-* ES cells by selection with 1.8 mg/ml of G418 (active weight) for 10-11 days.

Histological analysis of the gut epithelium

Irradiated chimeras were sacrificed 4.5 h after receiving 6 Gy of total body γ-irradiation. Fixation and multilabel immunohistochemical staining of chimeric intestine were as described[11]. TUNEL assays were performed on PLP-fixed tissues as previously described[22] except that slides were incubated in 0.5% Triton X-100/PBS for 20 min at room temperature.

Generation of *p21-/-* MEFs

Chimeras were generated by injection of 11-13 donor ES cells into C57B1/6 blastocysts[21]. 11 days after embryo injection and reimplantation ~E 13.5 embryos were harvested and MEFs isolated as described[23]. Passage 3 MEFs were selected with 300 μg/ml of G418 (Gibco BRL) for 6-7 days. p21 antiserum was raised against a GST-p21 fusion and depleted of anti-GST antibodies by affinity purification. Immunopreciptitations were as previously described[1].

Analysis of the irradiation response

MEFs were grown until subconfluency in DME, 10% FCS, 5 mM glutamine and antibiotics. Cells were plated at a density of $5\text{-}6*10^5$ cells per 10 cm plate and were irradiated between 24 and 30 hours after plating. Cell cycle analysis was as described[15]

with the exception that histones were extracted in 0.7% Triton-X 100, 1.2 N HC1. Samples were analyzed with a FACScan using CellQuest software (Becton-Dickinson). Immunoprecipitation and histone H1 kinase assays were performed as previously described[1].

ACKNOWLEDGMENTS

We thank G. J. Hannon in D. Beach's lab, for help at multiple stages of this project; C. Chandrasekaran in J.I. Gordon's lab for histological analysis of the chimeric intestines; M. Klemm, R. Jutterman and R. Jaenish for *p53-/-* ES cells; H. Hermeking for antibodies and E. Schmitt, G. Paradis, S. Salghetti, S. Matsumoto, S. Allan and J. Mkandawire for technical support. We thank R.A. Weinberg, A. Lassar, M. Serrano, H. Zhang and S. Lowe for helpful discussions and B. Williams, L.D. Attardi and K. Macleod for critical reading of the manuscript. This work was supported in part by grants from the N.I.H.

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Chapter 3

p21 Is a Critical CDK2 Regulator Essential for Proliferation Control in *Rb*-/- Cells

The work presented in this chapter is taken largely from a paper publised in *The Journal of Cell Biology* (Brugarolas et al. 1998). I would like to acknowledge R.T. Bronson for his help in the analysis of histological sections from tissues of *p21-/-;Rb+/-* mice.

ABSTRACT

Proliferation in mammalian cells is controlled primarily in the G1-phase of the cell cycle through the action of the G1 cyclin-dependent kinases, CDK4 and CDK2. To explore the mechanism of cellular response to extrinsic factors, specific loss of function mutations were generated in two negative regulators of G1 progression, p21 and pRB. Individually, these mutations were shown to have significant effects in G1 regulation, and when combined, *Rb* and *p21* mutations caused more profound defects in G1. Moreover, cells deficient for pRB and p21 were uniquely capable of anchorage-independent growth. In contrast, combined absence of pRB and p21 function was not sufficient to overcome contact inhibition of growth nor for tumor formation in nude mice. Finally, animals with the genotype *Rb+/-;p21-/-* succumbed to tumors more rapidly than *Rb+/-* mice, suggesting that in certain contexts mutations in these two cell cycle regulators can cooperate in tumor development.

INTRODUCTION

The hallmark of cancer is uncontrolled cell proliferation (for review see Sherr, 1996). Proliferation control, manifested by the integration and coordination of extracellular signals with the cell cycle machinery, is lost in tumor cells. Tumor cells proliferate autonomously; they are less dependent on growth-promoting signals and also less responsive to growth inhibitory signals. Numerous tissue culture assays have been developed that underscore these aspects of cellular transformation. For example, tumor cells grow at concentrations of growth factors insufficient for normal cell proliferation (Holley and Kiernan, 1968; Dulbecco, 1970; Jainchill and Todaro, 1970). They are also anchorage-independent for growth (MacPherson and Montagnier, 1964; Sanders and Burford, 1964) and less susceptible to contact inhibition than wild-type cells (Temin and Rubin, 1958; Todaro et al., 1963; Vogt and Dulbecco, 1963; Dulbecco, 1970).

The enzymes that regulate cell cycle progression, the cyclin-dependent kinases (CDKs) are candidates to integrate growth control signals with the cell cycle machinery (for review see Sherr, 1996). These enzymes, which are composed of a catalytic subunit and a regulatory subunit called cyclin, are regulated by multiple mechanisms, including the rate of synthesis, subcellular localization and degradation rate of the cyclin subunit (for review see Morgan, 1995). CDK activity is also regulated by stimulatory and inhibitory phosphorylation events (for review see Morgan, 1995) as well as by the binding of cyclin-dependent kinase inhibitors (CKIs) (for review see Peter, 1994). There are two families of CKIs, the Cip/Kip family and the InK4 family (for review see Sherr and Roberts, 1995). The Cip/Kip family consists of p21, p27 and p57 and is characterized by a conserved N-terminal CDK-binding domain, exclusive binding to heterodimeric complexes and affinity for multiple cyclin/CDK complexes. The InK4 family, consisting of p15, p16, p18 and p19, is distinguished by loosely conserved ankyrin motifs, binding to the catalytic kinase

subunit as well as the heterodimeric enzyme and exclusive association with CDK4 (or the CDK4 homolgue CDK6).

In mammalian cells, proliferation control is primarily achieved in the G1-phase of the cell cycle (Nilausen and Green, 1965; Todaro et al., 1965; Stoker et al., 1968; Otsuka and Moskowitz, 1975; Matsushisa and Mori, 1981). After G1, cells become largely independent of extracellular signals and progress automatically through subsequent cell cycle phases to the next G1 (Todaro et al., 1965; for review see Pardee, 1989). Hence, the G1 CDKs are likely to play a particularly important role in the integration of growth control signals with the cell cycle machinery. G1 progression is catalyzed by two enzymes: cyclin D (D1, D2 or D3)/CDK4 and cyclin E/CDK2 (for review see Sherr, 1994). Both enzymes regulate independent (Ohtsubo et al., 1995; Resnitzky and Reed, 1995) and essential (Baldin et al., 1993; Ohtsubo *et al.*, 1995; Pagano et al., 1993) events for G1 completion. Experimental manipulation of the activities of G1 CDKs by ectopic cyclin expression has revealed a critical role for these enzymes in the regulation of intrinsic G1 processes. For example, overexpression of cyclin D1 (Quelle et al., 1993; Resnitzky et al., 1994) or cyclin E (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994) shortens G1 and decreases the G1 cell size. Cyclin overexpression experiments suggest that G1 CDKs are also important in coupling extracellular signals with the cell cycle leading to reduced growth factor-dependence for proliferation (Ohtsubo and Roberts, 1993; Quelle et al., 1993). Importantly, while these observations underscore the importance of cyclins in the regulation of CDK activity, they do not address how this activity is regulated physiologically.

The CKI p21 (El-Deiry et al.; 1993; Gu et al.; 1993; Harper et al.; 1993; Xiong et al., 1993; Noda et al., 1994) may be a critical regulator of CDK activity. In normal cells, a significant fraction of cyclin/CDK complexes are found in quaternary complexes associated with p21 and PCNA (Zhang et al., 1994a). Kinase activity of cyclin/CDK

complexes may be dependent on p21 stoichiometry such that inhibition by p21 may require the association of more than one molecule of p21 per complex (Zhang et al., 1994b). Interestingly, quaternary complexes cannot be detected in many transformed cells (Xiong et al., 1993), suggesting that a p21 role in CDK regulation may be important in the regulation of the normal cell cycle.

Several lines of evidence suggest that p21 may have an especially important role in CDK2 regulation. In vitro, p21 has a very high affinity for cyclin E/CDK2 complexes (Gu et al., 1993) and greater than 95% of the active CDK2 in normal diploid fibroblasts is found associated with p21 (Harper et al., 1995). Additionally, in *Drosophila*, the p21/p27 homologue, Dacapo (Lane et al., 1996; Nooij et al., 1996), is responsible for cyclin E/CDK2 downregulation essential for arresting cells after endocycle 16 (Nooij et al., 1996). Finally, we have shown previously that p21 is necessary for CDK2 inhibition in response to p53 activation by γ-irradiation (Brugarolas et al., 1995).

The only essential function of cyclin D/CDK4 for G1 progression is the inactivation of the growth suppressive properties of the retinoblastoma protein, pRB. Although inhibition of cyclin D/CDK4 activity arrests cells with functional pRB, *Rb*-deficient cells are not arrested by such treatments (Lukas et al., 1994; Lukas et al., 1995). In its unphosphorylated form, pRB binds and inhibits the transcription factor E2F/DP (Chellappan et al., 1991). Upon phosphorylation, pRB releases E2F/DP, which then activates the expression of genes important for G1 completion and DNA replication (for review see Weinberg, 1995). Furthermore, overexpression of E2F1 is sufficient to drive quiescent cells into S-phase (Johnson et al., 1993). Although other proteins are known to associate with pRB in its unphosphorylated state, their significance in G1 progression is not clear (for review see Weinberg, 1995). The lack of requirement of *Rb-/-* cells for cyclin D/CDK4 suggests that these cells might not be responsive to growth control signals that act through cyclin D/CDK4. For example, exposing wild-type cells to low

concentrations of growth factors results in the decreased expression of D-type cyclins (Matsushime et al., 1991), and consequently decreased CDK4 activity, leading to G1 arrest (for review see Sherr, 1995). In contrast, *Rb-/-* cells are capable of growth in somewhat reduced concentrations of growth factors (Lukas et al., 1995). *Rb-/-* cells are not tumorigenic, however, indicating that they have not lost all the mechanisms of growth control (J.B. and T.J. unpublished data). One candidate to regulate proliferation in *Rb-/-* cells is cyclin E/CDK2. In fact, inhibition of cyclin E/CDK2 arrests cells irrespective of *Rb* status (Ohtsubo et al., 1995). Furthermore, in *Drosophila,* the ability of ectopically expressed E2F to drive cells into S-phase is dependent on cyclin E (Duronio et al., 1995). Thus, *Rb-/-* cells are likely to be susceptible to growth control signals that modulate CDK2 activity.

Here, we analyze the functions of p21 and pRB in the regulation of G1 and in the coupling of extracellular signals with the cell cycle. We have studied the role of p21 and pRB using mouse embryo fibroblasts (MEFs) that are deficient for these genes. The analysis of MEFs from knockout mice allows us to study the function of a specific gene, or subset of genes, in a very physiological context, using a primary cell population that has not accumulated other mutations, and with the rigor of tissue culture assays. For the experiments described here, we have generated *p21-/-*, *Rb-/-*, *Rb-/-;p21-/-* and wild-type MEFs from littermate embryos. The analysis of these cell types has revealed critical roles for p21 and pRB in G1 and in the regulation of pathways that are critical for the integration of growth control signals with the cell cycle machinery.

RESULTS

p21 is a physiological inhibitor of CDK2

In an effort to characterize the cell cycle effects of mutations affecting the CDK4 and CDK2 regulatory pathways, we first determined the consequences of *p21* mutation in primary cells in culture using a variety of assays. Although found in normal cells associated with multiple CDKs (Xiong et al., 1992; Harper et al., 1993), p21 does not bind all of them with equal affinity (Harper et al., 1995), suggesting differential regulation by p21. In vitro, p21 has a very high affinity for complexes containing CDK2 and CDK4 (Harper et al., 1995). To examine the role of p21 in the regulation of these G1 CDKs, we determined CDK4 and CDK2 kinase activities in exponentially growing *p21-/-* and wild-type control MEFs generated from littermate embryos. Protein extracts were prepared and precleared of nonspecific kinase activity, and CDK4 and CDK2 immunoprecipitates were incubated in the presence of ATP-γ^{32}P and a vast excess of GST-RB as a kinase substrate. As shown in Fig. 1A, the catalytic activity of CDK4 was very similar in *p21-/-* and wild-type cells. Therefore, in exponentially growing fibroblasts, p21 does not appear to be a major CDK4 inhibitor. In contrast, CDK2 activity in *p21-/-* cells was elevated 2-4 fold compared to wild-type cells (Fig. 1B, C). This increased activity in *p21*-deficient cells could not be accounted for by increased levels of cyclin E, cyclin A or CDK2 (data not shown). Furthermore, more than 50% of CDK2 in wild-type MEFs was found associated with p21 (data not shown), providing support to the idea that the effect of p21 loss on CDK2 activity is direct. Thus, in exponentially growing fibroblasts in tissue culture, p21 is a critical inhibitor of CDK2.

pRB is phosphorylated by CDK2 containing complexes both in vitro and in vivo (for review see Weinberg, 1995); pRB phosphorylation results in a retarded migration in

SDS-PAGE. Western blot analysis of pRB from extracts of wild-type and *p21-/-* cells normalized for protein content revealed an increase in the slow migrating form of pRB in *p21-/-* as compared to wild-type cells (Fig. 1D), suggesting that pRB is phosphorylated to a greater extent in *p21-/-* than in wild-type cells. This increase in pRB phosphorylation is likely to reflect the increased CDK2 activity observed in *p21-/-* as compared to wild-type cells in the in vitro CDK2 kinase assays.

p21-/- cells show altered G1 regulation

Because *p21-/-* cells have higher levels of CDK2 activity than wild-type cells, we sought to determine whether they would exhibit a decreased G1 length as has been shown for cells overexpressing cyclin E (Ohtsubo and Roberts, 1993). Cultures of *p21-/-* and wild-type MEFs were labeled with the thymidine analog 5-bromodeoxiuridine (BrdU) for 5 hours, fixed, stained with the DNA intercalating agent propidium iodide (PI), and assayed by two dimensional FACS analysis. Table I shows the results from four independent experiments. The analysis of the distribution of *p21-/-* and wild-type cells showed that the percentage of mutant cells in G1 compared to wild-type cells was modestly decreased by 5.2% (Table I). These data suggest that the length of G1, compared to the overall cell cycle length, is shorter in *p21-/-* cells than in wild-type cells.

Elevated levels of CDK2 activity have also been shown to reduce the G1 cell size (Ohtsubo and Roberts, 1993), which might be a consequence of the G1 shortening. To analyze the size of *p21-/-* cells, asynchronously growing *p21-/-* and wild-type cells were fixed, stained with PI, and analyzed by FACS. The PI staining allowed us to correlate DNA content with cell size. Comparative analysis of the cell size between wild-type and *p21-/-* cells revealed that *p21-/-* cells contained a larger percentage of cells with smaller size (assessed by forward scatter, FSC-H) (Fig. 2A). Note that FSC-H gives an indication of

diameter and that small differences in diameter represent larger differences in volume. The cell size distribution of *p21-/-* cells with a 2n DNA content (i.e. in G1) was shifted towards the left of that of wild-type cells (Fig. 2B) accounting for the increased number of smaller cells observed in Fig. 2A. Interestingly, despite clear differences in G1 cell size, the size distribution of *p21-/-* and wild-type cells with a 4n DNA content was very similar (Fig. 2E). Thus, between the beginning of S-phase and prior to cytokinesis, some of the *p21-/-* cells would seem to increase in size to a larger extent than wild-type cells, apparently compensating for the smaller 2n cell size.

Additive effects of the *Rb* and *p21* mutations in the G1-phase

The phenotype of *p21-/-* cells is reminiscent of the phenotype that has been previously reported for *Rb-/-* MEFs (Lukas et al., 1995; Herrera et al., 1996). To compare the role of p21 and pRB in the regulation of G1, we generated *Rb-/-* and *p21-/-* littermate embryos from *p21+/-;Rb+/-* crosses and derived MEFs from them. These cells were then analyzed in the assays described above. *Rb-/-* MEFs have similar levels of CDK4 and CDK2 kinase activities to wild-type MEFs (Lukas et al., 1995; Fig. 1). The analysis of the cell cycle phase distribution of *Rb-/-* cells indicated a pattern remarkably similar to that of *p21-/-* cells (Table I). Previously, *Rb*-deficient MEFs were shown to be smaller than wild-type MEFs (Herrera et al., 1996; Fig. 2A); we have found that this decrease in size is due to a decrease in the size of G1 cells (Fig. 2B). As with *p21-/-* cells, the size distribution of cells with a 4n DNA content was similar between *Rb-/-* and wild-type cells (Fig. 2E).

Rb-/- cells contain several fold higher levels of cyclin E compared to wild-type cells, which is thought to reflect increased E2F activity (Herrera et al., 1996). This increased cyclin E, paradoxically, does not result in a proportional increase in cyclin E associated CDK2 activity, although the activity is elevated in *Rb-/-* cells in G1 (Herrera et

al., 1996). We have found that *Rb-/-* cells also contain several fold higher levels of p21 as compared to wild-type cells (Fig. 1D). This increase in p21 may also reflect increased E2F transcriptional activity as E2F-1 has been shown to specifically transactivate the *p21* promotor (Hiyama et al., 1997). Thus, increased p21 levels may result in the downregulation of CDK2 activity and could explain why cyclin E associated CDK2 activity does not increase proportionally to cyclin E levels.

Next we examined whether combined dysregulation of CDK2 (through mutation of *p21*) with disruption of CDK4 (through *Rb* mutation) pathways would cause additional G1 phase defects. Constitutive activation of these two pathways through these mutations might also be expected to limit the ability of cells to stop the cell cycle machinery in response to extracellular growth inhibitory signals. To test these possibilities, we generated embryos deficient in both genes and isolated MEFs from them. *p21-/-;Rb-/-* embryos were generated from intercrosses of *p21-/-;Rb+/-* mice. From these crosses, however, all resulting MEF populations were homozygous mutant for *p21*. Therefore, to generate wild-type, *Rb-/-* as well as *p21-/-* control embryos from the same litter as the *p21-/-;Rb-/-* mutants, we also intercrossed a large series of *p21+/-;Rb+/-* animals.

Rb-/-;p21-/- cells (similar to *p21-/-* cells) had 2-4 fold higher levels of CDK2 activity compared to *Rb-/-* or wild-type cells (Fig. 1B and C). Comparative analysis of the cell cycle distributions of *Rb-/-;p21-/-* cells indicated a further reduction in the percentage of cells in G1; while the number of cells in G1 in *p21-/-* and *Rb-/-* cells was decreased by 5.2% and 5% respectively, it was reduced by 11.6% in double mutant cells (Table I). Conversely, the double mutant cells showed an increase in the percentage of cells in S-phase and G2/M that was approximately twice that seen in comparing single mutant to wild-type cells (Table I). These data indicate that loss of pRB and p21 have additive effects in G1-phase regulation. Cell size analysis revealed that the cell size distribution of *Rb-/-;p21-/-* cells is shifted towards the left of that of single mutant cells (Fig. 2A). This seems

to be due to an increase in the proportion of smaller G1 cells (Fig. 2C). Because of the relatively small changes in cell diameter, we analyzed the size of *Rb-/-;p21-/-* cells derived from two different embryos. As shown in Fig. 2D, the size of cells from these two strains was indistinguishable. As observed for the single mutant cells, the size distribution of *Rb-/-;p21-/-* cells with a 4n DNA content is similar to wild-type cells (Fig. 2E).

We have further characterized G1 progression in *Rb-/-;p21-/-* cells by analyzing the cell cycle profiles of G0 synchronized double mutant and wild-type cells as they progress to S-phase. As shown in Fig. 3A-F, double mutant cells enter S-phase 3-4 hours earlier than wild-type cells. Interestingly, however, the reduced G1 length was not associated with a significant change in the population doubling time. When compared in standard growth curve analysis, double mutant cells accumulated with similar kinetics to *Rb-/-*, *p21-/-* and wild-type cells (Fig. 3G). These data suggest that the decrease in G1 length does not lead to a reduction in overall cell cycle length, perhaps due to compensatory effects in the S and G2/M phases; such effects would be indicated from the cell size analysis described above.

Decreased serum-dependence for proliferation of *Rb-/-;p21-/-* cells

G1 CDKs are candidates to integrate extracellular signals with the cell cycle. Thus, mutations in constitutive regulatory elements of these pathways may impair the cell's ability to regulate the cell cycle machinery in response to extracellular signals. To test this possibility, we analyzed the effects of loss of pRB and p21 in serum responsiveness. The growth properties of *Rb-/-;p21-/-*, *p21-/-*, *Rb-/-* and wild-type MEFs were characterized in the presence of limiting quantities of serum. Cells were synchronized in G0 and plated at low density in 0.1% FCS and 0.5% FCS. Duplicate samples were collected on five consecutive days and counted. As expected, no increase in cell number occurred in wild-type cells in 0.1% FCS and only a very small increase was observed in 0.5% FCS (Fig. 4A

and B). *p21-/-* cells were capable of some proliferation at both serum concentrations, as were *Rb-/-* cells (Fig. 4A and B). Strikingly, *Rb-/-;p21-/-* cells had a significant growth advantage over either single mutant strain, as well as wild-type cells (Fig. 4A and B). Similar results were obtained in 0.05% and 0.3% FCS (data not shown). *Rb-/-;p21-/-* cells however, still exhibited a significant dependence on serum for proliferation as observed by comparing the growth rate of these cells in 0.5% and 5% FCS (Fig. 4C). It is important to note that these data do not demonstrate that p21 and pRB are direct regulators of the serum response pathway, only that mutations in constitutive regulators of G1 CDK pathways affect the ability of cells to arrest in the presence of concentrations of growth factors insufficient for normal cell proliferation.

Loss of pRB and p21 is sufficient for anchorage-independent growth

Fibroblast cell proliferation is contingent upon adhesion and spreading onto an adequate substratum (Green and Nilausen, 1962; for review see Assoian, 1997). Plating cells in semisolid medium results in a G0/G1 arrest (Otsuka and Moskowitz, 1975; Matsushisa and Mori, 1981; Guadagno and Assoian, 1991) that is thought to be mediated through the downregulation of CDK4 and CDK2 activities (for review see Assoian, 1997). Because *Rb-/-;p21-/-* cells should not require cyclin D/CDK4 activity for proliferation and exhibit 2-4 fold higher levels of CDK2 activity than wild-type cells, we investigated whether these cells retain the ability to arrest in non-adherent conditions. *Rb-/-;p21-/-* MEFs from two different embryos were assayed for anchorage-independent growth in soft agar. *p21-/-*, *Rb-/-* and wild-type MEFs derived from littermate embryos were used as controls. Cells of each genotype were plated onto dishes coated with 3% low melting point (LMP) agarose in media containing 20% FCS and allowed to grow for three weeks. Cultures were supplemented with 0.3% LMP agarose in media containing 20% FCS every 3 days. While

the different control cells failed to grow under these conditions (Fig. 5 A-C, and E-G), *Rb-/-;p21-/-* were capable of forming colonies (Fig. 5D and H). Colony size varied but some colonies were quite large (Fig. 5H). These data demonstrate that loss of both pRB and p21 is sufficient to allow anchorage-independent growth, while the presence of either inhibitor singly still allows growth arrest.

Plating cells on standard agarose prevents cell spreading and allows the retrieval of cells for biochemical analysis (Guadagno et al., 1993). Fibroblasts plated under these conditions downregulate cyclin D1 (Bohmer et al., 1996; Zhu et al., 1996) (the major D-type cyclin in fibroblasts (Won et al., 1992)) and inhibit cyclin E/CDK2 activity (Fang et al., 1996; Zhu et al., 1996). Cyclin E/CDK2 inhibition is not caused by downregulation of cyclin E levels or by inhibitory phosphorylation of CDK2 (Fang et al., 1996), but instead is thought to be mediated by p21 and p27 (Fang et al., 1996; Zhu et al., 1996). However, p21 and p27 were only somewhat induced in anchorage-independent conditions (Fang et al., 1996; Zhu et al., 1996); increased levels of p21 and p27 bound to cyclin E/CDK2 may result from an increase in the pool of free inhibitors due to the decreased levels of cyclin D1/CDK4 complexes (for review see Assoian, 1997).

Similar to 3T3 fibroblasts, MEFs responded to anchorage-independent conditions with a reduction in cyclin D1 levels (Fig. 6A) and an inhibition of CDK2 (Fig. 6C and D). As with 3T3 cells, the levels of p21 and p27 in MEFs did not change significantly under these conditions (Fig. 6B and data not shown), suggesting again that CDK2 inhibition may be due to a redistribution of the CKIs. In an effort to understand the molecular mechanisms that underlie the ability of *Rb-/-;p21-/-* cells to grow in soft agar, we characterized CDK activity biochemically. Protein extracts from *Rb-/-;p21-/-* and wild-type control cells in monolayers and in suspension were normalized for protein content and used for western blot analysis and kinase assays. As shown in Fig. 6, double mutant MEFs downregulated cyclin D1 and CDK2 activity under these conditions. However, *Rb-*

/-;p21-/- cells failed to downregulate CDK2 activity to wild-type levels (Fig. 6C and D). In fact, the levels of CDK2 activity in non-adherent *Rb-/-;p21-/-* cells were very similar to the levels present in exponentially growing wild-type cells. The level of CDK2 activity observed in double mutant cells in semisolid medium can only be partially accounted for by loss of p21, however, because CDK2 activity in *p21-/-* cells under the same conditions was found to be 2-4 fold higher than in wild-type cells (Fig. 6E), compared to the 4-6 fold higher levels observed in the double mutants (Fig. 6C, D and E). The remaining increase in CDK2 activity in *Rb-/-;p21-/-* cells could therefore reflect the increased proportion of proliferating cells in these cultures.

Rb-/-;p21-/- cells are susceptible to contact inhibition of growth

Normal fibroblasts grow in monolayers and arrest with a 2n DNA content at high cell densities (Nilausen and Green, 1965). This arrest is thought to involve p27 (Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994), although loss of p27 does not alter the susceptibility of MEFs to contact inhibition (Nakayama et al., 1996). Thus, other molecules in addition to p27 must be involved in this response. Having shown that *Rb-/-;p21-/-* cells do not arrest in conditions preventing anchorage, we next tested the susceptibility of these cells to contact inhibition of growth. *Rb-/-;p21-/-*, wild-type, *p21-/-* and *Rb-/-* MEFs derived from littermate embryos were plated at $2x10^6$ cells per 10 cm dish and allowed to grow to confluence for 6 days. Cells were then harvested, fixed, stained with PI and evaluated by FACS analysis. As shown in Fig. 7, all cultures exhibited a prominent G0/G1 peak indicative of a cell cycle arrest. The mutant cultures had a somewhat higher S-phase and G2/M fractions, suggesting a slightly reduced capacity for contact-inhibition. Thus, in contrast to anchorage independence, loss of pRB and p21 is not sufficient to overcome growth inhibitory signals triggered by cell-cell contact.

Given the slight increase in the S-phase or G2/M fractions among the mutant cells at confluence, we examined these cells using a more stringent test for contact inhibition: the focus-formation assay. Mutant cells were plated amongst wild-type cells at a ratio 1:100 and cultures were scored at 4 weeks for the presence of foci. Neither *Rb-/-* or *Rb-/-;p21-/-* cells were capable of forming foci under these conditions (data not shown). Thus, by this measure as well, combined elimination of p21 and pRB failed to allow escape from contact inhibition.

We have shown that the capacity of *Rb-/-;p21-/-* cells to sustain anchorage-independent growth correlates with failure to downregulate CDK2 activity to wild-type levels. Because *Rb-/-;p21-/-* cells are susceptible to contact inhibition of growth, they might be able to inhibit CDK2 under these conditions. To test this hypothesis, CDK2 kinase activity was assayed from protein extracts prepared from contact-inhibited and asynchronously exponentially growing *Rb-/-;p21-/-* and wild-type MEFs. As shown in Fig. 8B and C, *Rb-/-;p21-/-* cells at high densities downregulated CDK2 activity to wild-type levels. Furthermore, under these conditions, we detected a several-fold increase in p27 levels in wild-type and double mutant cells (Fig. 8A), suggesting that p27 upregulation may contribute to the inhibition of CDK2 at high cell densities in both cell types.

Rb-/-;p21-/- cells do not form tumors in nude mice

Growth in soft agar usually correlates with tumorigenic potential (Freedman and Shin, 1974; for review see Assoian, 1997). Thus, to assay the tumorigenicity of *Rb-/-;p21-/-* cells, $2x10^6$ MEFs were injected subcutaneously into nude mice. *p53-/-* cells expressing the viral oncogene *E1A* and an activated *ras* allele (T24 H-ras) (Lowe et al., 1994) were used as a positive control. While, mice injected with *p53-/-;E1A;ras* transformed cells developed tumors within two weeks, no tumors were evident after 6 months in mice

injected with *Rb-/-;p21-/-* cells (data not shown).

Decreased tumor survival rates of *p21-/-;Rb+/-* mice

As another means to address the potential cooperative tumorigenic effects of *p21* and *Rb* mutations, we have characterized the tumor phenotype of animals with the genotype *p21-/-;Rb+/-*. *Rb+/-* mice have a strong tumor predisposition (Jacks et al., 1992; Williams et al., 1994), with a mean age of survival around 340 days on a mixed genetic background (C57BL/6-129/Sv) (Williams et al., 1994). These mice develop intermediate lobe pituitary and medullary thyroid adenomas and adenocarcinomas (Jacks et al., 1992; Williams et al., 1994), and tumors show loss of heterozygozity at the *Rb* locus (Williams et al., 1994). In addition to these tumors, chimeric mice composed of wild-type and *Rb-/-* cells also develop pheochromocytomas, indicating that the *Rb* mutation can also predispose to this tumor type (Williams et al., 1994). In contrast, *p21-/-* mice do not develop tumors (Deng et al. 1995) by 1.5 years of age (data not shown). Absence of p21 did not alter the tumor spectrum caused by the *Rb* mutation, as *p21-/-;Rb+/-* mice also seemed to develop exclusively pituitary tumors, medullary thyroid adenomas and adenocarcinomas and pheochromocytomas (Table II). Interestingly, the mutation of *p21* did have a significant effect on the lifespan of animals heterozygous for an *Rb* mutation. As shown in Fig. 9 and Table II, the mean age of survival of *p21-/-;Rb+/-* mice was 261 days compared to 340 days for *Rb+/-* mice. Although subtle differences in the balance of 129/Sv versus C57BL/6 alleles in these mixed genetic background animals could contribute to this effect, it is likely that absence of p21 increases the transformation potential or growth properties of *Rb-/-* cells in certain tissues, perhaps in a manner analogous to the effects reported here for fibroblasts in culture. Moreover, similar tumor size at necropsy between *Rb+/-* (MAS=340 d) and *p21-/- ;Rb+/-* (MAS=261 d) mice further supports the contention that

loss of p21 can accelerate tumor development in *Rb*+/- mice.

Figure 1. Elevated CDK2 activity in *p21*-deficient cells.

(*A*) In vitro CDK4 kinase assay using GST-RB as a substrate. (*B*) In vitro CDK2 kinase assay using GST-RB as a substrate. (*C*) Histogram representing quantitative analysis of the results from the CDK2 kinase assay. (*D*) Western blot analysis of p21 and pRB in wild-type, *p21*-/- and *Rb*-/- cells. Lanes shown were reassembled in appropriate order from a single autoradiogram. All samples were derived from the same experiment.

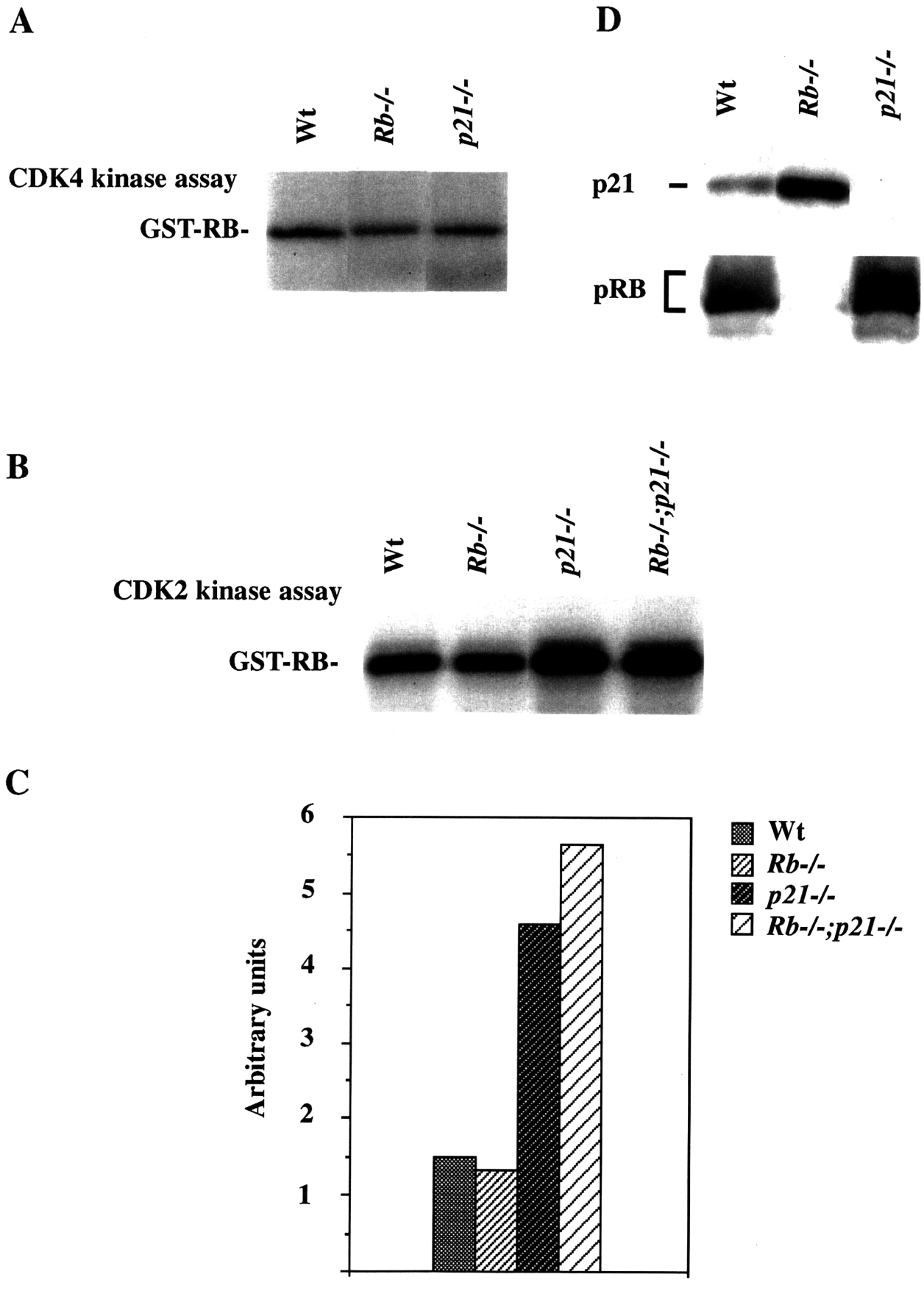
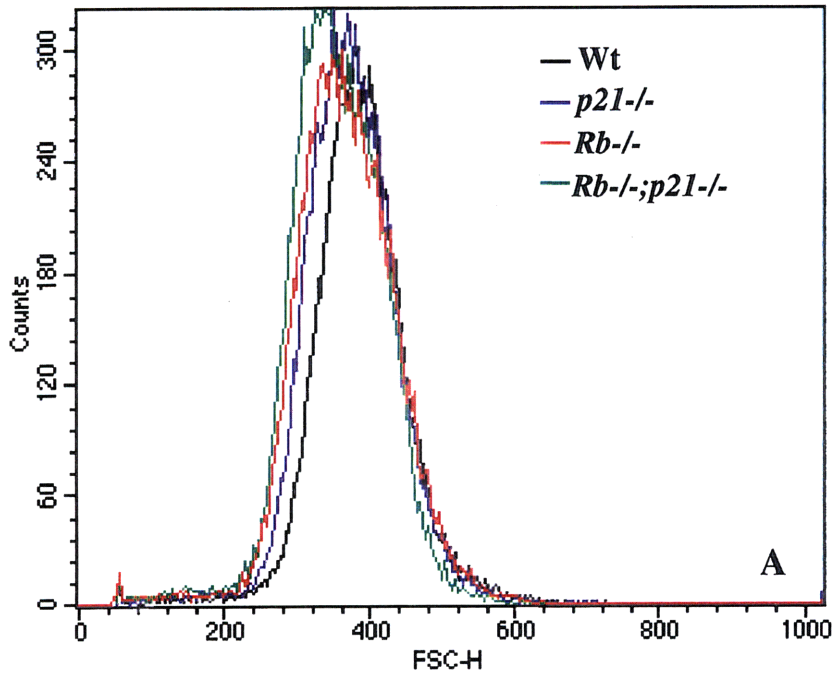
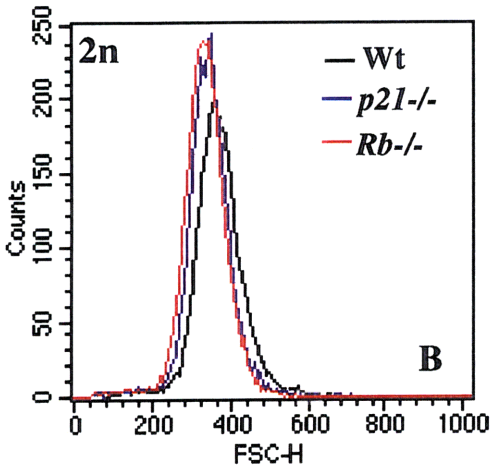
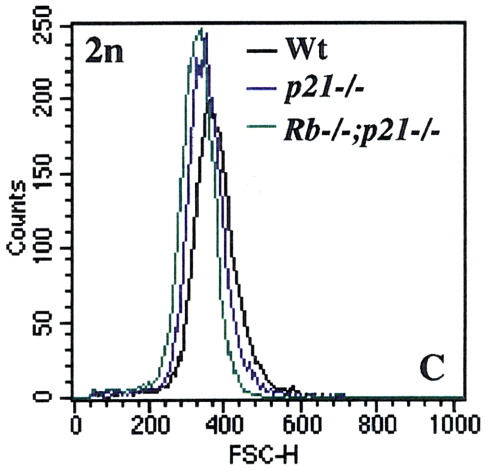
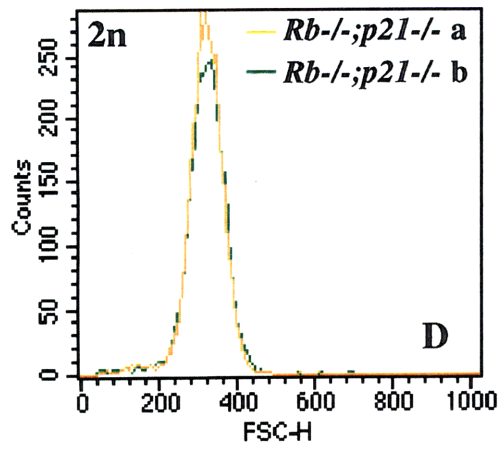

A
Wt
Rb-/-
p21-/-
CDK4 kinase assay
GST-RB-
D
Wt
Rb-/-
p21-/-
p21
pRB
B
Wt
Rb-/-
p21-/-
Rb-/-;p21-/-
CDK2 kinase assay
GST-RB-
C
Arbitrary units
6
5
4
3
2
1
Wt
Rb-/-
p21-/-
Rb-/-;p21-/-

Figure 2. Cell size analysis of wild-type, *p21-/-*, *Rb-/-* and *Rb-/-;p21-/-* cells. Asynchronously growing cells were harvested, fixed, stained with PI and analyzed by FACS. 60,000 events were collected for each sample. 2n and 4n DNA containing populations were gated for cell size analysis in a FSC-H by FL2-A dot plot and scored in a FSC-H histogram. (*A*) Cell size analysis of wild-type, *p21-/-*, *Rb-/- Rb-/-;p21-/-* cells. (*B*, *C*) Cell size analysis of wild-type, *p21-/-*, *Rb-/-*, *Rb-/-;p21-/-* cells with a 2n DNA content. (*D*) Size analysis of two strains of *Rb-/-;p21-/-* cells with a 2n DNA content. These strains were derived from two different embryos. (*E*) Cell size analysis of wild-type, *p21-/-*, *Rb-/-* and *Rb-/-;p21-/-* cells with a 4n DNA content.


Wt
p21-/-
Rb-/-
Rb-/-;p21-/-
Counts
FSC-H
A


2n
Wt
p21-/-
Rb-/-
Counts
FSC-H
B


2n
Wt
p21-/-
Rb-/-;p21-/-
Counts
FSC-H
C


2n
Rb-/-;p21-/- a
Rb-/-;p21-/- b
Counts
FSC-H
D

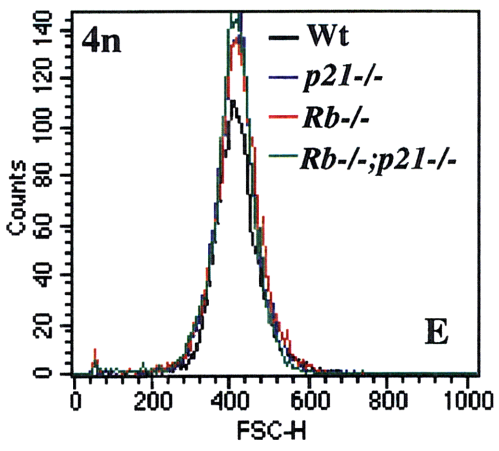
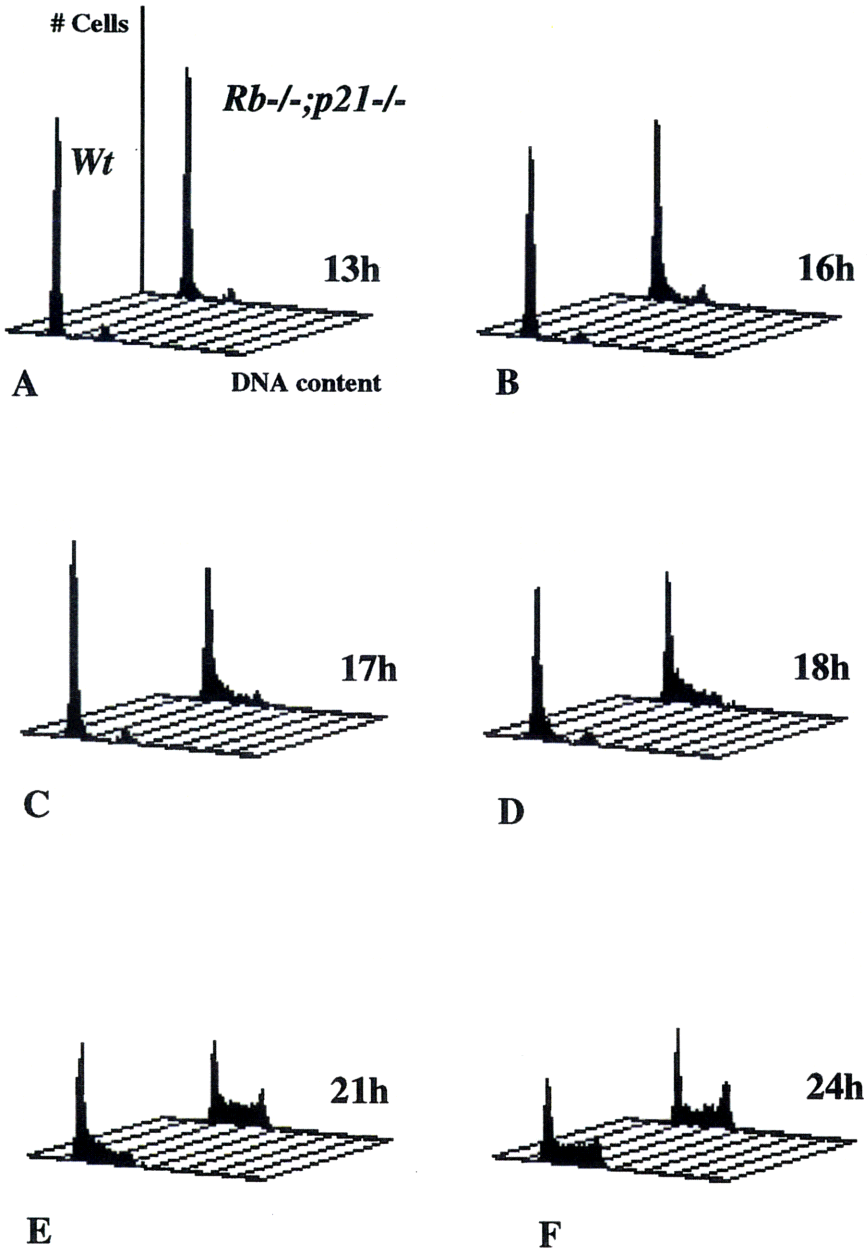

4n
Wt
p21-/-
Rb-/-
Rb-/-;p21-/-
Counts
FSC-H
E

Figure 3. Growth rate and cell cycle analysis of G0-S phase interval of wild-type, *p21-/-*, *Rb-/-* and *Rb-/-;p21-/-* cells.

(*A-F*) DNA content profiles of synchronous cultures of wild-type and *Rb-/-;p21-/-* cells at different times after release from G0. (*G*) Growth properties of *Rb-/-;p21-/-*, *Rb-/-*, *p21-/-* and wild-type MEFs derived from littermate embryos in tissue culture media supplemented with 10% FCS. Values at different timepoints represent the average of the measurements from duplicate samples. This experiment was performed three times with duplicate samples with similar results.


Cells
Rb-/-;p21-/-
Wt
13h
16h
A
DNA content
B
17h
18h
C
D
21h
24h
E
F

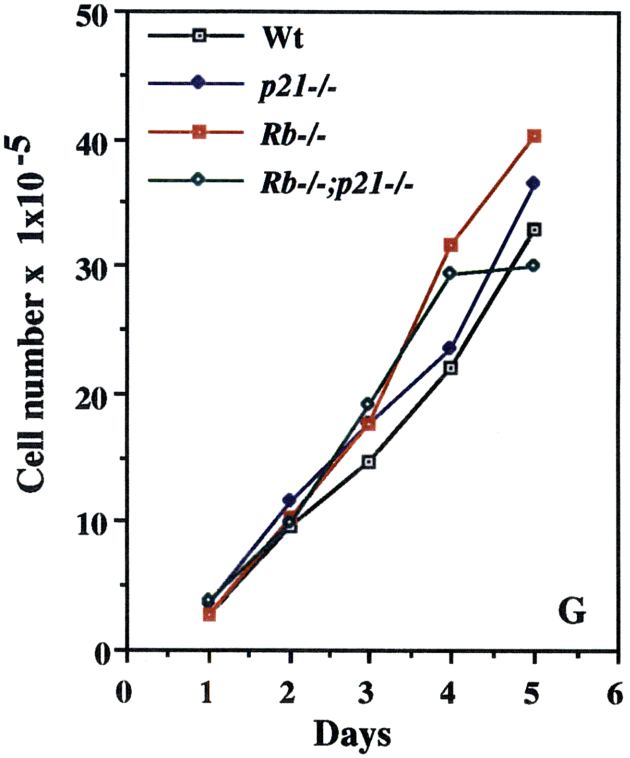

Wt
p21-/-
Rb-/-
Rb-/-;p21-/-
Cell number x 1x10-5
Days
0
10
20
30
40
50
0
1
2
3
4
5
6
G

Figure 4. Decreased serum-dependence for proliferation of mutant cells.

(*A* and *B*) Cells were synchronized in G0 and replated at low density in 0.1% FCS or 0.5% FCS. At the indicated times, duplicate samples were harvested and counted. Similar results were obtained at 0.05% FCS and 0.3% FCS (data not shown). (C) Comparative analysis of the growth properties of *Rb-/-;p21-/-* cells in 0.5% and 5% FCS.

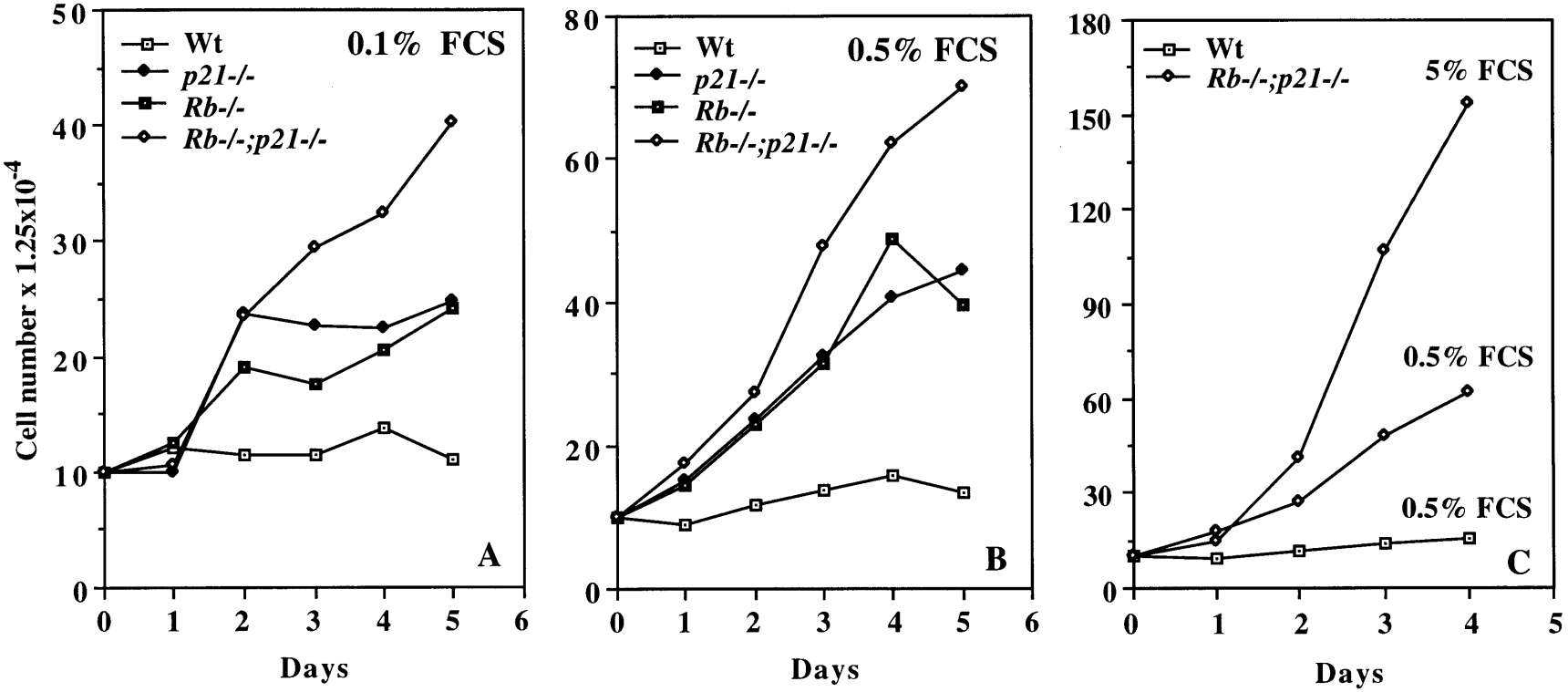
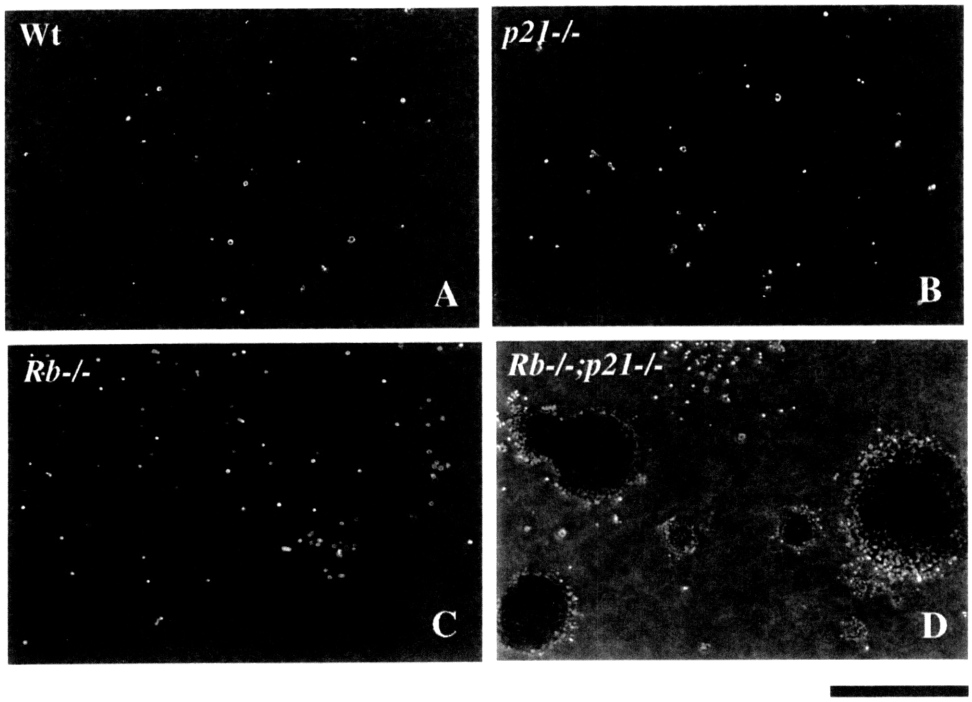

Cell number x 1.25x10^{-4}
Wt
p21-/-
Rb-/-
Rb-/-;p21-/-
0.1% FCS
A
Days
0.5% FCS
B
5% FCS
0.5% FCS
0.5% FCS
C

Figure 5. *Rb-/-;p21-/-* cells are anchorage-independent for proliferation.

G0 synchronized populations were plated in soft agar and colony formation was scored after three weeks. Bars: (*A-D* and *E-H*) 500 μm.


Wt
A
p21-/-
B
Rb-/-
C
Rb-/-;p21-/-
D

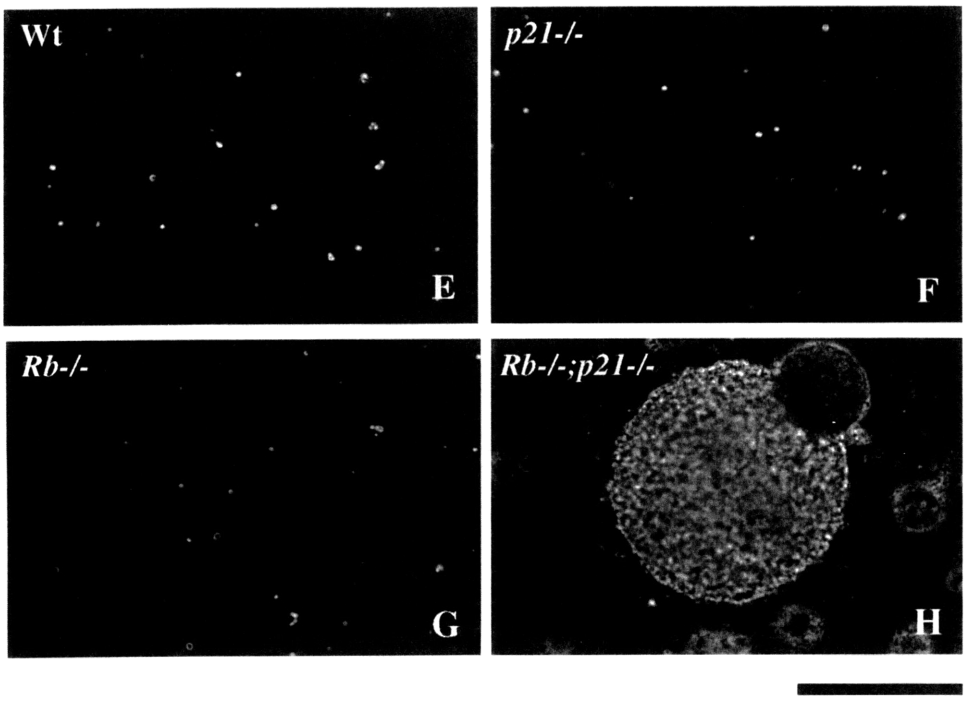

Wt
E
p21-/-
F
Rb-/-
G
Rb-/-;p21-/-
H

Figure 6. Biochemical analysis of *Rb-/-;p21-/-* cells grown in monolayer (Mn.) and in suspension (Sp.).

(*A*) Cyclin D1 protein levels assayed by western blot analysis. (*B*) p27 protein levels assayed by western blot analysis. (*C*) In vitro CDK2 kinase assay ussing GST-RB as a substrate. (*D*) Histogram representing quantitation analysis of the results from the CDK2 kinase assay. (*E*) In vitro CDK2 kinase assay of wild-type, *p21-/-*, and *Rb-/-;p21-/-* cells in suspension using GST-RB as a substrate. Lanes shown were reassembled in appropriate order from a single autoradiogram. All samples were derived from the same experiment.

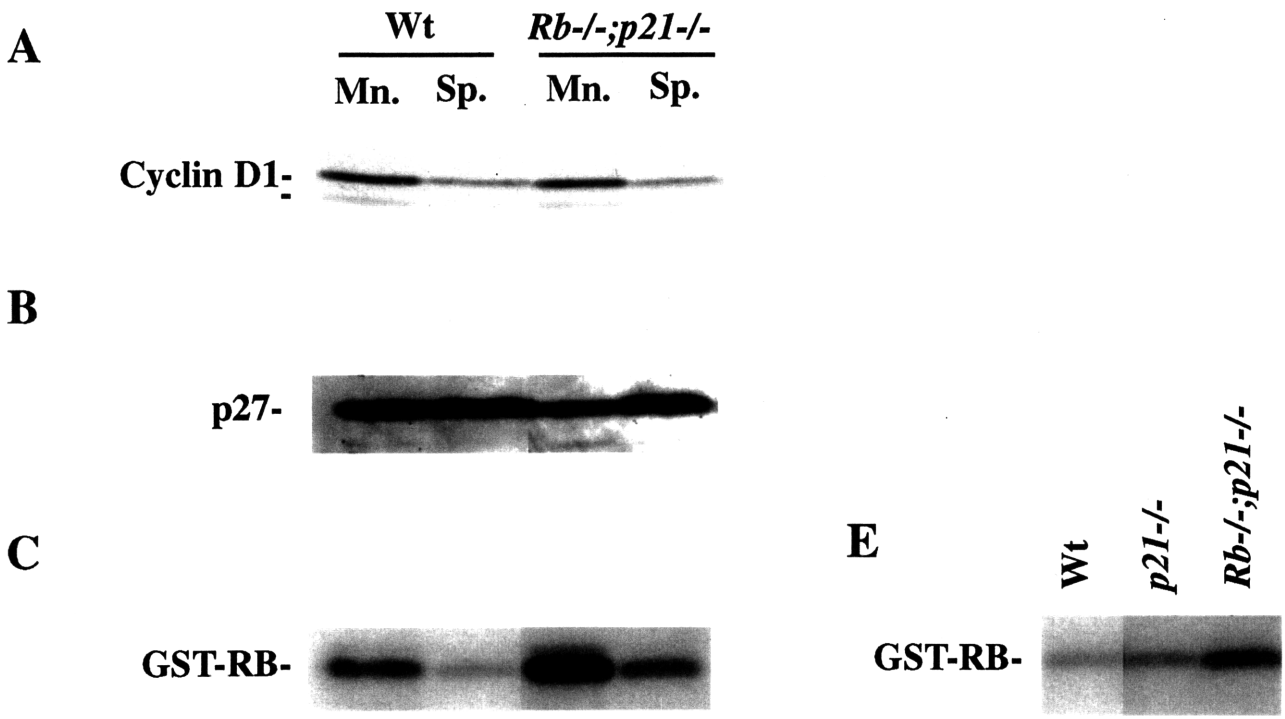
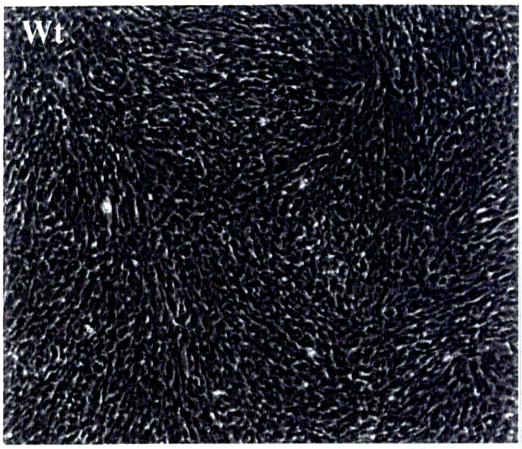
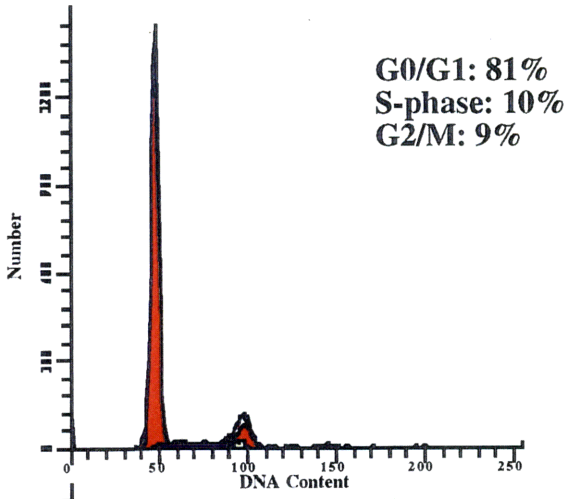
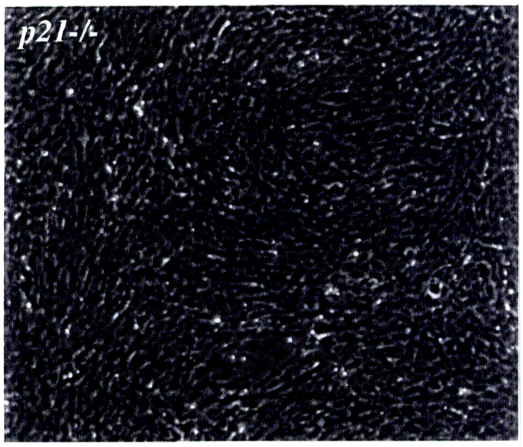

A
Wt
Rb-/-;p21-/-
Mn.
Sp.
Mn.
Sp.
Cyclin D1-
B
p27-
C
GST-RB-
E
Wt
p21-/-
Rb-/-;p21-/-
GST-RB-
D
Arbitrary units
4
3
2
1
Wt Mn.
Wt Sp.
Rb-/-;p21-/- Mn.
Rb-/-;p21-/- Sp.

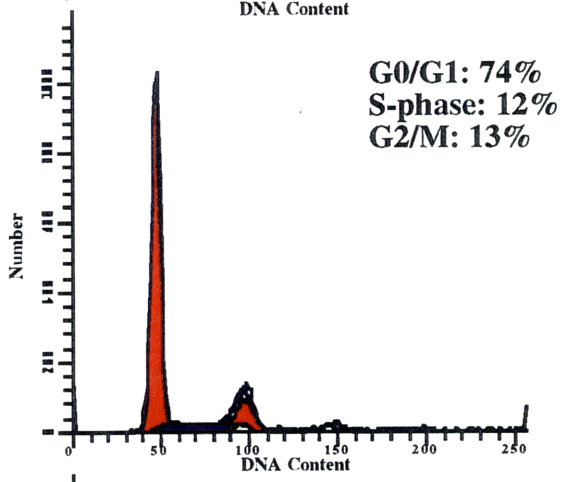
Figure 7. *Rb-/-;p21-/-* cells are susceptible to contact inhibition of growth.

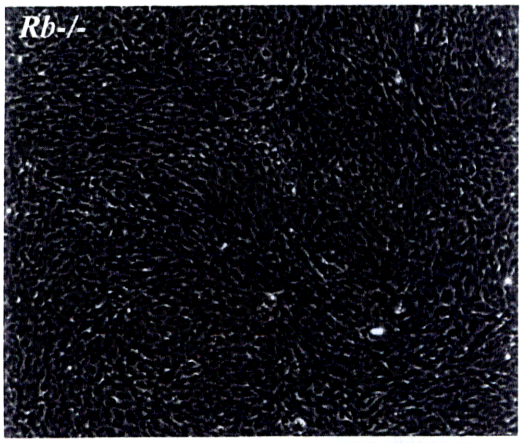
The left column shows representative fields of confluent cultures. The right column shows cell cycle profiles of confluent cultures after 6 days. 10,000 events were collected for each sample. The data were analyzed using ModFit LT software (Becton Dickinson). The coefficient of variation was below 6 for all the samples. Bar, 500 μm.

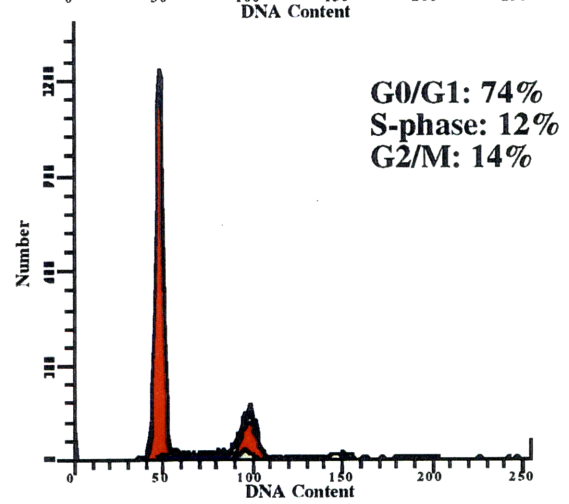

Wt


G0/G1: 81%
S-phase: 10%
G2/M: 9%
Number
DNA Content


p21-/-


G0/G1: 74%
S-phase: 12%
G2/M: 13%
Number
DNA Content


Rb-/-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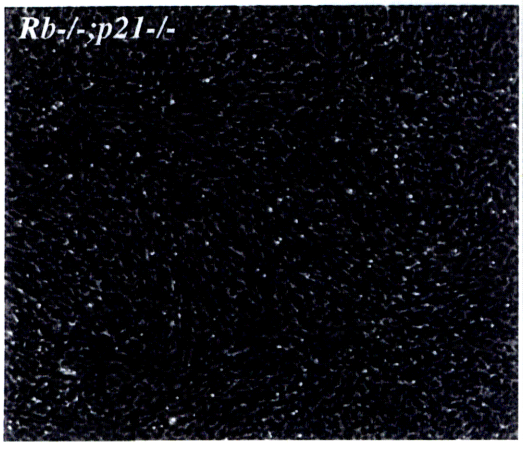
S-phase: 12%
G2/M: 14%
Number
DNA Content


Rb-/-;p21-/-

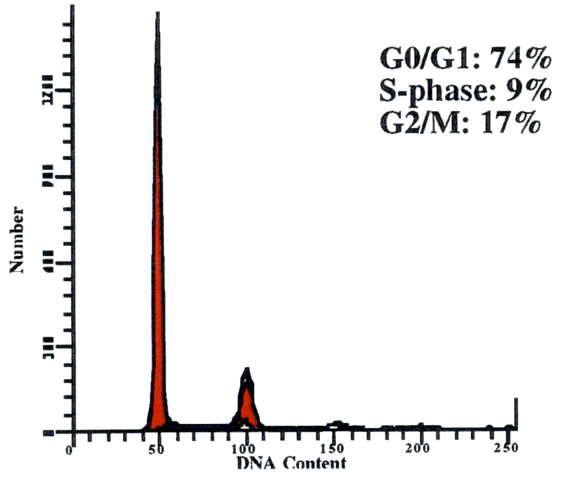
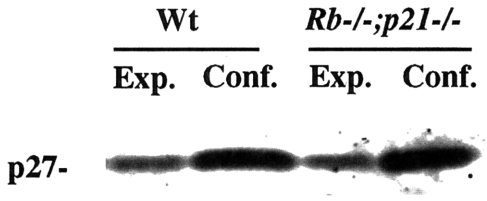

G0/G1: 74%
S-phase: 9%
G2/M: 17%
Number
DNA Content

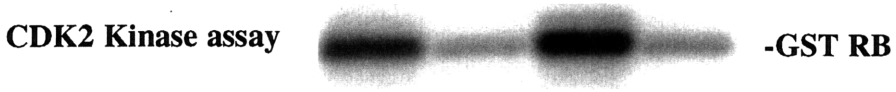
Figure 8. Biochemical analysis of exponentially growing (Exp.) and confluent (Conf.) *Rb-/-;p21-/-* cells.

(*A*) p27 protein levels analyzed by western blot analysis. (*B*) In vitro CDK2 kinase assay using GST-RB as a substrate. (*C*) Histogram representing quantitative analysis of the results from the CDK2 kinase assay.

A


Wt
Rb-/-;p21-/-
Exp.
Conf.
Exp.
Conf.
p27-

B


CDK2 Kinase assay
-GST RB

C

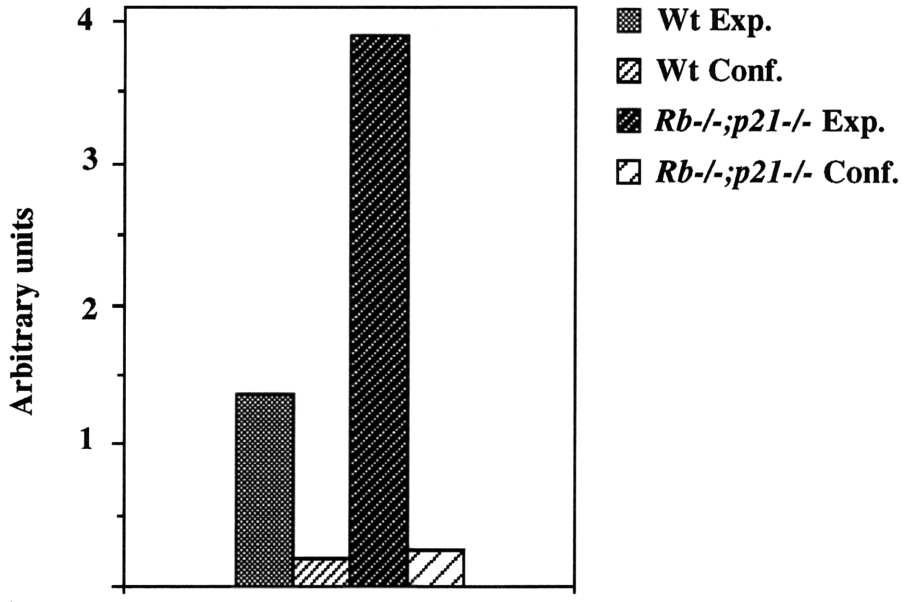

4
3
2
1
Arbitrary units
Wt Exp.
Wt Conf.
Rb-/-;p21-/- Exp.
Rb-/-;p21-/- Conf.

Figure 9. Survival curve of *p21*-/-;*Rb*+/- mice.

Comparison of *p21*-/-;*Rb*+/- mice (n=70) to *Rb*+/- mice (n=50) (Williams et al., 1994) in a mixed C57BL/6-129/Sv genetic background.

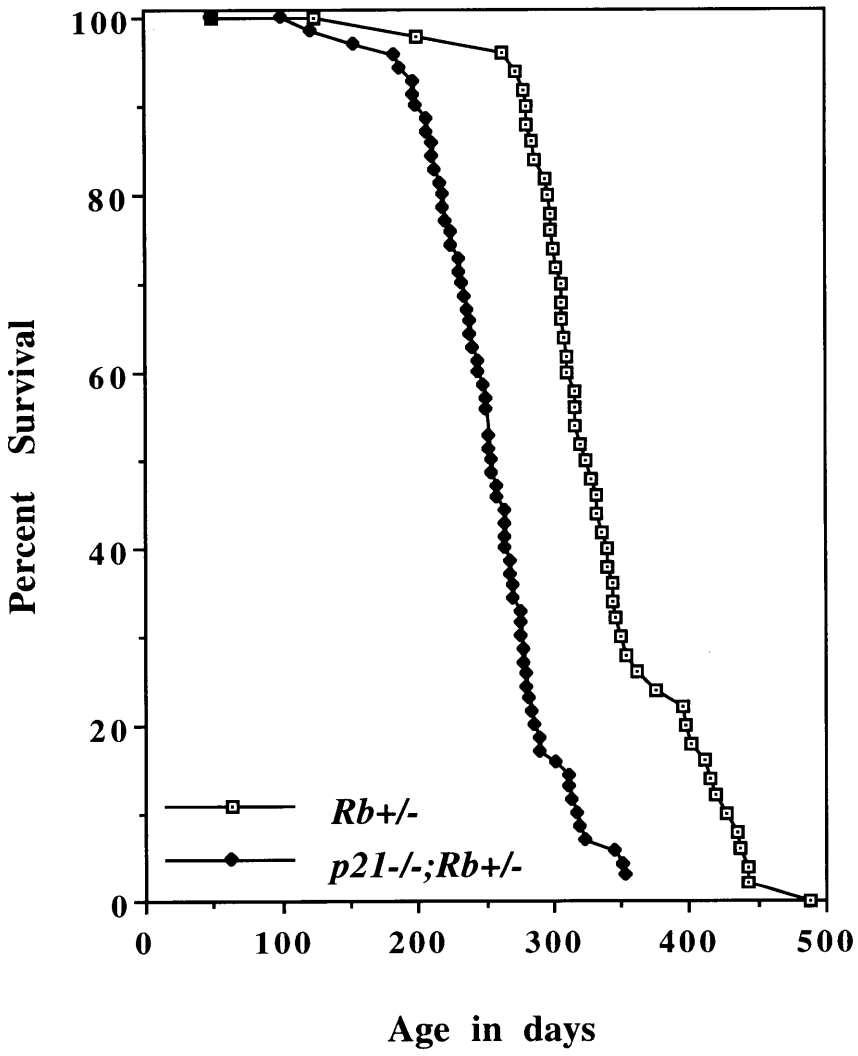

100
80
60
40
20
0
Percent Survival
0
100
200
300
400
500
Age in days
Rb+/-
p21-/-;Rb+/-

Table I. Cell Cycle Distribution

	%G1	%S-phase	%G2/M
Wt	**47.2+/-0.6**	**41+/-0.9**	**11.8+/-0.9**
p21-/-	**42+/-2.2**	**44.5+/-2.1**	**12.8+/-1.1**
Rb-/-	**42.2+/-4.4**	**45.5+/-4.7**	**12.6+/-1.1**
Rb-/-;p21-/-	**35.6+/-2.8**	**50.8+/-2.8**	**13.6+/-0.4**

Analysis of the cell cycle distribution of asynchronously growing wild type, *p21-/-*, *Rb-/-* and *Rb-/-;p21-/-* cells. Cells were pulsed with 5-Bromodeoxiuridine (BrdU) for 5 hours, fixed, stained with PI and analized by 2D FACS analysis. The data shows the average of 4 independent experiments and standard deviations of the measurements.

Table II. Tumor spectrum of *p21-/-;Rb+/-* mice.

	Rb+/-	***p21-/-;Rb+/-***
M.A.S.	**340 (n=50)**	**261 (n=57)**
Pituitary tumor	**200/200**	**27/27**
Medullary thyroid tumor	**19/27**	**23/26**
Pheochromocytoma	**N.D.**	**7/19**

Tumor analysis in *p21-/-;Rb+/-* mice compared to *Rb+/-* mice (Williams et al., 1994).

M.A.S. (Mean age of survival).

DISCUSSION

In this manuscript, we describe the use of specific loss-of-function mutations in two key inhibitors of the mammalian cell cycle to explore the mechanism of cellular response to extrinsic factors that regulate cell cycle progression. Previous studies using cyclin overexpression or p27 antisense have demonstrated that pathways regulated by the G1 CDKs, CDK4(6) and CDK2 are important in this process (Ohtsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994; Coats et al, 1996; Rivard et al, 1996). Our approach has been to systematically eliminate functions that regulate or are regulated by these two pathways in an attempt to render the cell insensitive to conditions that normally elicit cell cycle arrest. From these experiments, we hope to clarify the normal cellular response to growth inhibitory conditions as well as to investigate the relationship between the loss of normal cell cycle regulation and neoplastic transformation. Taken together, our data support a model in which regulation of G1 progression can be divided into two discrete stages controlled by the activities of CDK4(6) and CDK2, such that inhibition of either stage can prevent cell cycle progression. Thus, in cells in which the CDK4(6) pathway is constitutively activated through *Rb* mutation and CDK2 pathway is upregulated through *p21* mutation, neither growth factor limitation nor detachment from the substratum results in proper cell cycle arrest.

Although capable of anchorage-independent growth, *p21-/-;Rb-/-* cells are still growth arrested at high cell densities. The difference in response to these two conditions was correlated with the ability of the double mutant cells to downregulate CDK2 activity to wild-type levels. The more complete inhibition of CDK2 activity at confluence may be explained by the observed induction of p27, which was not seen when the cells were prevented from contacting the substratum. Interestingly, *p27*-deficient MEFs downregulate CDK2 kinase activity to wild-type levels (A.Koff, personal communication)

and arrest at confluency (Nakayama et al., 1996). Perhaps either p21 or p27 is sufficient for CDK2 regulation under these conditions. This issue can be explored further by constructing *p21/p27* double-mutant MEFs.

Defects in the regulation of growth in tissue culture have been used as a measure of neoplastic transformation (for review see Ponten, 1976; Smets, 1979), with anchorage-independent growth correlating well with tumorigenicity (Freedman and Shin, 1974; reviewed by Assoian, 1997). Interestingly, despite their ability to form colonies in soft-agar, *p21-/-;Rb-/-* cells were not tumorigenic in nude mice. Thus, the loss of these two inhibitors is not sufficient for proliferation in vivo. In contrast to our observations, the combination of ras activation and *p21* mutation is sufficient for transformation of mouse skin keratinocytes, including the ability to form tumors in nude mice (Missero et al., 1996). We believe that tumor formation in nude mice by ras transformed *p21*-deficient keratinocytes but not *Rb-/-;p21-/-* MEFs reflects critical oncogenic functions of activated *ras* beyond its effects on pRB (Peeper et al., 1997).

As indicated by the reduced life span of *p21-/-;Rb+/-* mice, germline mutations in these two genes can synergize in tumor development. By analogy with our fibroblast data, this effect could be a reflection of elevated CDK2 activity potentiating the growth of *Rb-/-* tumor cells. It appears that the loss of *Rb* function dictates the tumor spectrum, because we observed the same tumor types in double mutant as in *Rb+/-* animals. In contrast, germline mutations in *Rb* and *p53* produce a broader tumor spectrum than either of the single mutants (Williams et al., 1994; Harvey, 1995). Therefore, while p21 is a key downstream target of p53 in growth arrest, it appears that the major tumor suppressor function(s) of p53 do not require p21. The acceleration of tumorigenesis in *p21-/-;Rb+/-* mice indicates a context in which p21 function does limit tumor cell growth, and could represent another mechanism of tumor suppression by p53; this model would assume that p53 is the major regulator of p21 expression in some tumor types.

The integrity of the CDK4 and CDK2 pathways is not only critical in the regulation of cell cycle progression in response to extracellular signals but also in response to intrinsic cues and senescence. Thus, p21 and pRB are required for the integrity of the p53-mediated G1 arrest response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995; Slebos et al., 1994), and loss of p21 (J.B., T.J. unpublished data) or INK4a (Serrano et al., 1996) greatly enhance the life span of MEFs in tissue culture. In addition, Serrano et al., (1997) have recently reported that overexpression of the *ras* oncogene can induce a senescence-like state, in a manner dependent on both INK4a and p53 function. Therefore, mutations in the regulatory elements of the CDK4 and CDK2 (Keyomarsi and Pardee, 1993; Kitahara et al., 1995; Porter et al., 1997) pathways may not only be sufficient to overcome extracellular growth inhibitory signals but also intracellular growth inhibitory signals and senescence, and it might be expected that regulatory elements of these pathways would be critical targets for tumorigenesis (for review see Hirama and Koeffler, 1995).

Tumor cells proliferate autonomously, independently of growth inhibitory signals and despite the absence of growth-promoting signals. Thus, tumor cells are defective in the integration of extracellular signals with the cell cycle machinery. These defects lie at multiple levels, in cell surface receptors, signal transduction pathways and cell cycle regulators. We have studied the role of two inhibitors of G1 progression in this process. Our data support the conclusion that proliferation control is primarily accomplished in G1 and that p21 and pRB are critical components of these regulatory pathways. We have shown that in the absence of pRB, CDK2 can act as the gatekeeper that secures some degree of proliferation control. We postulate that constitutive activation of the CDK4 and CDK2 pathways is sufficient for unrestrained proliferation and may render cells unable to execute a senescence triggered arrest. A better understanding of the mechanisms that coordinate extracellular growth control signals with the cell cycle machinery in the normal cell is of primary importance to understand how growth autonomy is achieved in cancer.

EXPERIMENTAL PROCEDURES

MEF generation and cell culture

MEFs were generated from 12.5 post-coitum (pc) mouse embryos. Embryos were harvested, the brain and internal organs were removed and the carcasses were minced and incubated with trypsin for 30-45 minutes at 37^{o}C. Tissue culture media (DME supplemented with 10% heat inactivated FCS (IFS), 5mM glutamine and penicillin/streptomycin) was added to the cell suspension and the cells were further disaggregated. Homogeneous cell suspensions were added to T75 flasks containing 20 ml of media. After 3-4 days in an incubator at 37^{o}C , confluent cultures were trypsinized and replated onto 2 T175 flasks containing 50 ml of tissue culture media. After 3 days cells were frozen at 2-3x10^{6} cells per vial in DME supplemented with 10% IFS and 10% DMSO. Cells were frozen at -80^{o}C and stored at -150^{o}C. Vials were subsequently thawed and cells expanded every 3 days. Cells were seeded at 1.5-2x10^{6} cells per 10 cm dish or equivalent. Early passage (p<6) MEFs were used for all the experiments.

Genotyping

PCR to detect *Rb* status was performed as described previously (Jacks et al., 1992). The following primers were used to determine the *p21* genotype: 5' AAG CCT TGA TTC TGA TGT GGG C 3' (for both the wild-type and the mutant allele), 5' TGA CGA AGT CAA AGT TCC ACC G 3' (specific to the wild-type allele) and 5' GCT ATC AGG ACA TAG CGT TGG C 3' (specific to the mutant allele). 10x PCR buffer: 500 mM KCl, 100 mM Tris (pH 8.3), 15 mM MgCl, 1mg/ml BSA, 2 mM dNTPs. Thermocycling: Step 1: 4 minutes at 94^{o}C; Step 2: 40 cycles of 1 minute at 72^{o}C, 1 minute at 64^{o}C and 3 minutes at 72^{o}C; Step 3: 7 minutes at 72^{o}C. Polynucleotides were separated in a 2% agarose gel with the wild-type being ~ 900 bp and the mutant band being ~750 bp.

G0 synchronization

1.5-2x10^6 MEFs were plated in 10 cm dishes and grown to confluency for 4 days in media supplemented with 10%IFS. Fibroblasts were washed with PBSA and incubated for an additional 4 days in media supplemented with 0.1% IFS.

Cell cycle and cell size analysis

Asynchronously growing cells were washed with PBSA, trypsinized and fixed in 70% methanol at -20°C for several hours. Cells were centrifuged at 2000 rpm and resuspended in PBS containing RNase A (Sigma) at 0.1mg/ml. Samples were incubated at 37°C for 15-30 minutes and PI (Sigma) was added to final concentration of 0.2 mg/ml. After 6 hours at 4°C, samples were processed by a FACScan (Becton-Dickinson). Data was analyzed with ModFit LT software (Becton-Dickinson). Cell size analysis was performed on 2n and 4n DNA containing populations gated from a FSC-H/FL2-A dot plot and represented in a FSC-H histogram. To analyze the cell cycle distribution, cells were pulsed for 5 hours with 0.3 μg/ml 5-bromodeoxiuridine (Sigma) and 0.03 μg/ml 5-fluoro-5-deoxiuridine (Sigma). Samples were processed as described in Brugarolas *et al.*, 1995.

Soft agar assays

50,000 cells synchronized in G0 were resuspended in 0.34% low melting point agarose (LMP agarose) (Gibco BRL) in DME supplemented with 20% IFS. Cells were plated onto 6 cm dishes coated with 0.5% LMP agarose in DME plus 20% IFS. Cultures were maintained in an incubator at 37°C and were supplemented with 2 ml of 0.34% LMP agarose in DME containing 20% IFS every three days . Samples were analyzed for colony formation at three weeks.

Suspension cultures

Suspension cultures were performed as described previously (Guadagno and Assoian, 1991), with some modifications. $3.5x10^6$ G0 synchronized cells were resuspended in media supplemented with 20% IFS and plated onto agarose coated plates (0.8% agarose (Gibco BRL) in DME supplemented with 20% IFS). Cells were harvested for protein extracts after 3 days using a rubber policeman.

Focus formation Assay

Cells from two different strains of *Rb-/-;p21-/-* and *Rb-/-* MEFs were seeded with wild-type MEFs in 10 cm dishes at a ratio 1:100. Media was changed every three days, and the cultures were followed for four weeks.

Immunoblotting

Protein extracts were prepared as described (Zhu et al., 1996). Protein concentration was evaluated with the BioRad protein assay. 300 µg of protein were fractionated by SDS-PAGE and transferred to Immobilon-P (Millipore) or nitrocellulose (NitroPlus). Membranes were blocked in TBS-T (10 mM Tris (pH 7.5), 150 mM NaCl, 0.03% Tween-20) containing 5% non-fat dry milk. Cyclin D1 was detected using a mouse anti-cyclin D1 antibody (Santa Cruz, HD-11) at 0.5 µg/ml, a secondary rabbit-against-mouse IgG antibody (Jackson Immmunoresearch) at 0.2 µg/ml and a tertiary anti-rabbit IgG antibody conjugated to HRP (Amersham) at a dilution 1:6000. p27 detection was performed using a goat anti-p27 antibody (Santa Cruz, C-19-G) at 0.5 µg/ml, a secondary mouse-anti-goat IgG antibody (Jackson Immunoresearch) at 0.2 µg/ml and a tertiary antibody against-mouse IgG conjugated to HRP (Amersham) at 1:6000. P21 and pRB were detected using Santa Cruz C-19-G and Pharmingen G3-245 antibodies respectively. Detection was performed by chemiluminescence.

In vitro kinase assays

CDK2 and CDK4 *in vitro* kinase assays were performed as described previously (Matsushime et al., 1994) with the following modifications. Cell lysates (between 180-450 μg of protein were used for CDK2 kinase assays and between 0.8-1.3 mg of protein were used for CDK4 kinase assays) were precleared with equilibrated protein A beads (Pierce) and incubated with anti-CDK4 (Santa Cruz, C-22) or anti-CDK2 (kindly provided by G. J. Hannon) for 4 hours. Immune complexes were precipitated with protein A beads (Pierce) and incubated in the kinase buffer containing 4 mM ATP, 20 μCi [γ-^{32}P] ATP (NEN-Dupont) and 6 μg of GST-RB (GST fusion with aminoacids 792-928 from the C-terminus of pRB) or 2 μg of histone H1 (Sigma), for 30-60 minutes at 30°C. Quantitation was performed by phosphorimager analysis.

Tumorigenicity assays

Fibroblasts were trypsinized, washed with PBSA and resuspended at 1x10^7 cells/ml in PBSA. 2x10^6 cells were injected subcutaneously into two flanks of three 5 week old Swiss nu/nu mice (Taconic). Mice were monitored for 6 months.

Pathology analysis

Over 20 *Rb*+/-;*p21*-/- mice were examined histologically for tumors in the CNS, retina, pituitary gland, thyroid gland, salivary gland, trachea, lungs, heart, stomach, small and large intestine, liver, pancreas, spleen, testis, prostate, ovaries, uterus, mammary gland, skin, bone, kidneys and adrenals. Blood samples were also collected for analysis.

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Chapter 4

Inhibition of CDK2 by p21 Is Necessary for pRB-mediated G1 Arrest Following γ-Irradiation

The work presented in this chapter is taken largely from a paper submitted to *Genes and Development* (Brugarolas et al. 1998, submitted). Figure 3A in this chapter was contributed by K. Moberg.

ABSTRACT

In mammalian cells, DNA damaging agents result in a G1 arrest that is dependent upon the tumor suppressor, p53, and its transcriptional target, p21. Using primary cell lines lacking specific cell cycle regulators, we demonstrate that this pathway functions through the growth suppressive properties of the pRB tumor suppressor. Specifically, γ-irradiation inhibits the phosphorylation of pRB at cdk2- but not cdk4-specific sites in a p21-dependent manner. Most importantly, we show that pRB is an critical component of this DNA damage checkpoint. These data indicate that the p53 $\Rightarrow$ p21 checkpoint pathway uses the normal cell cycle regulatory machinery to induce the accumulation of the growth suppressive form of pRB.

INTRODUCTION

The retinoblastoma gene (*Rb*) was originally cloned by virtue of its absence in retinoblastomas (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). Subsequent studies showed that *Rb* gene mutations exist in approximately one third of all human tumors (for review, see Weinberg 1992). These mutations result in either complete loss or functional inactivation of the retinoblastoma protein and reintroduction of the wild-type gene is sufficient to reverse the tumorigenicity of several *Rb* negative cell lines (Huang et al. 1988; Bookstein et al. 1990).

The growth suppressive properties of pRB are thought to be dependent upon its ability to regulate the cellular transcription factor E2F (for review, see Nevins 1992). The retinoblastoma protein binds to E2F *in vivo* (Bagchi et al. 1991; Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991) and this association is sufficient to inhibit its transcriptional activity (Hiebert et al. 1992). Moreover, the resulting pRB•E2F complex is capable of mediating the transcriptional repression of E2F-responsive genes (Weintraub et al. 1992; Bremner et al. 1995; Weintraub et al. 1995). Many E2F-responsive genes have been identified and they each play a critical role in the control of cellular proliferation (for review, see Nevins et al. 1997). In addition, overexpression of E2F causes cell cycle entry (Johnson et al. 1993). Finally, E2F binding maps to the "growth suppression" domain of the retinoblastoma protein (Qin et al. 1992) and mutant, tumor derived-forms of pRB all lack the ability to bind to E2F (Bandara and La Thangue 1991; Chellappan et al. 1991; Kaelin et al. 1991; Hiebert et al. 1992).

The retinoblastoma protein is phosphorylated in a cell cycle dependent manner and this modifications are sufficient to inactivate its ability to bind to E2F and to block cell division (for review, see Dyson and Harlow 1992). Several pRB phosphorylation sites have been identified and they each match the consensus recognition sequence of the cyclin-

dependent kinases (cdks; Lees et al. 1991). The G_1 cdks, cyclin D•cdk4/6 and cyclin E•cdk2, can both phosphorylate pRB *in vitro* (Lees et al. 1991; Hinds et al. 1992; Hu et al. 1992; Ewen et al. 1993; Matsushime et al. 1994; Meyerson and Harlow 1994). In these *in vitro* assays, cyclin D•cdk4 and cyclin E•cdk2 preferentially phosphorylate distinct but overlapping pRB sites (Connell-Crowley et al. 1997; Zarkowska and Mittnacht 1997). Cyclin D•cdk4 is the first cdk to be activated in response to growth factors (Matsushime et al. 1994) and *in vivo* studies confirm that it is essential for pRB inactivation (Lukas et al. 1995a; Lukas et al. 1995b). Indeed, pRB appears to be the only essential target of this kinase (Lukas et al. 1995a; Lukas et al. 1995b). Studies addressing the role of cyclinE•cdk2 in pRB regulation have yielded conflicting conclusions (Connell-Crowley et al. 1997; Ezhevsky et al. 1997; Lundberg and Weinberg 1998). One study demonstrated that cyclin D1/cdk4 is sufficient to inactivate both the E2F binding and growth suppressive properties of pRB (Connell-Crowley et al. 1997). However, two other labs have reported that inhibition of cdk2 (by either treatment with TGFβ or over-expression of dominant negative cdk2) results in the accumulation of an under-phosphorylated form of pRB that can still bind to E2F (Ezhevsky et al. 1997; Lundberg and Weinberg 1998). These latter studies suggest that cyclinE•cdk2 will contribute to inactivation of the growth suppressive properties of pRB. In contrast to the D-type kinases, cyclinE•cdk2 is known to have at least one other substrate whose phosphorylation is essential for S-phase entry (Ohtsubo et al. 1995).

Superimposed on the normal cell cycle regulation are a number of checkpoint mechanisms. These are not required for normal cell cycle progression but are critical for the cellular response to stress (Paulovich et al. 1997). One of the best characterized of the mammalian checkpoint pathways is the DNA damage-induced G_1 arrest. This checkpoint is dependent upon the tumor suppressor, p53 (Kastan et al. 1992). The loss of p53 abrogates the DNA damage response and this is thought to contribute to tumorigenesis by

permitting the propagation of DNA mutations (for review, see Lane 1992). The mechanism by which p53 imposes the DNA-damage induced G_1 arrest has been partially elucidated. In response to irradiation, p53 induces the transcription of the *p21* gene (El-Deiry et al. 1993), which encodes an inhibitor of cyclin-dependent kinases (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993). Analysis of *p21*-deficient cells, confirms that the p21 protein is essential for the integrity of the DNA damage-induced G_1 arrest (Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995). In normal cells, this p21-dependent arrest correlates with the accumulation of hypo-phosphorylated pRB (Dulic et al. 1994; Slebos et al. 1994). Although this change in pRB phosphorylation could arise as an indirect consequence of the G_1 arrest, the presence of the pRB-binding, papilloma virus E7 protein has been shown to abrogate the DNA damage checkpoint (Slebos et al. 1994). These data suggest that pRB is involved in the DNA damage response, however, the multifunctional nature of E7 makes it difficult to eliminate other possible mechanisms (Funk et al. 1997; Jones et al. 1997).

In this study, we investigate how the p53 $\Rightarrow$ p21 DNA damage checkpoint pathway interfaces with the normal cell cycle machinery. Using cell lines derived from mutant mouse strains, we demonstrate that the p21-mediated arrest is dependent upon the accumulation of active, growth suppressive pRB. Induction of the checkpoint by low doses of γ-irradiation, results in the downregulation of cdk2, but not cdk4, kinase activity and the accumulation of partially phosphorylated pRB. Phosphopeptide specific antibodies confirm that this form of pRB has been phosphorylated on cdk4- but not cdk2-specific sites. Thus, p21 arrests cells by blocking the inactivation of the retinoblastoma protein that normally occurs as cells progress through the G_1 phase of the cell cycle. Our data supports a model whereby this checkpoint blocks cell cycle progression by co-opting normal cell cycle regulatory mechanisms.

RESULTS

γ-irradiation of fibroblasts results in the activation of the DNA damage checkpoint pathway and thereby induces cell cycle arrest (Kastan et al. 1991). It is well documented that p21 acts as an important downstream target of this p53-dependent radiation response but the mechanism of p21 action is unknown (Dulic et al. 1994; Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995). Since the pRB-binding, papilloma virus E7 protein is sufficient to over-ride the DNA damage checkpoint (Slebos et al. 1994), we tested if pRB is required for the p53 ⇒ p21 mediated arrest. To address this question, we generated primary mouse embryo fibroblasts (MEFs) from wild-type, *p53-/-*, *p21-/-* and *Rb-/-* mouse strains and tested their response to DNA damage. Wild-type and mutant cells were exposed to γ-irradiation and the degree of G_1 arrest was assessed by comparing the proportion of S-phase cells in irradiated versus unirradiated populations (Fig. 1). Consistent with previous studies, loss of p53 dramatically impairs the G_1 cell cycle block. In contrast, loss of p21 significantly reduced, but did not abolish, this γ-irradiation induced arrest. This supports previous conclusions that p21 is a critical downstream target of p53 but it is not the only mechanism by which p53 can mediate the DNA damage-induced G_1 arrest (Brugarolas et al. 1995; Deng et al. 1995).

pRB is necessary for the DNA damage checkpoint

When tested in this assay, *Rb*-deficient fibroblasts were also impaired in their ability to arrest in G_1 in response to DNA damage. Significantly, the magnitude of this defect was similar to that observed in the *p21-/-* cells, suggesting that p21 and pRB act in the same DNA damage response pathway. To test this hypothesis, we generated mouse strains that were deficient for both *p21* and *Rb* and then compared the irradiation response of single

and double mutant MEFs derived from littermate embryos (Fig. 1). The G1 arrest response of the *p21-/-;Rb-/-* cells was indistinguishable from that of either of the single mutant MEFs. Therefore, we conclude that p21 and pRB act downstream of p53 in the same checkpoint pathway.

CDK2 inhibition by p21 following irradiation

We hypothesized that p21 acts upstream of pRB to block its cell cycle dependent phosphorylation. This model is supported by the finding that p21 can inhibit the activity of the known pRB kinases, cyclin D•cdk4 and cyclin E•cdk2 (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993), and by reports indicating that these kinases are downregulated in response to a variety of DNA damaging agents (Terada et al. 1995; Poon et al. 1996). To test the role of kinase inhibition in our system, we compared the level of cdk4 and cdk2 kinase activity in wild-type and *p21*-deficient cells either with or without γ-irradiation treatment (Fig. 2A). We did not detect any significant effect of γ-irradiation on the level of cdk4 kinase activity in either cell type. In contrast, γ-irradiation dramatically reduced the level of cdk2 kinase activity in wild-type but not *p21-/-* cells. These data strongly suggest that the p21-dependent, radiation induced G_1 arrest is mediated through the specific inhibition of cdk2 but not cdk4 kinase activity.

Regulation of pRB phosphorylation in wild-type and *p21*-deficient cells following irradiation

To test the role of pRB phosphorylation in this DNA damage response, we immunoprecipitated pRB from wild-type (downregulated cdk2 kinase) and *p21-/-* (normal cdk2 kinase) irradiated cells that had been metabolically labeled with $^{32}P_i$ (Fig. 2B).

Consistent with the continued presence of cdk4 kinase activity (see Fig. 2A), we detected phosphorylated pRB in extracts of wild-type irradiated cells (Fig. 2B). This phosphorylated pRB was present in two distinct forms, a slower migrating species (labeled band A) and a faster migrating species (labeled band B), that were detected at roughly equal levels. Both of these pRB species were also present in the irradiated p21-deficient cells. In these cells, however, the slower migrating form of pRB (band A) was present at much higher levels than the faster migrating form (band B). This suggested that γ-irradiation results in a change in the degree of pRB phosphorylation in a p21-dependent manner.

To assess the relative levels of each pRB species (instead of the degree of ^{32}P incorporation), we also performed immunoprecipitation followed by immuno-blotting of whole extracts of wild-type and *p21-/-* irradiated cells (Fig. 2C). In this assay, we were able to detect three distinct pRB species. The fastest migrating form (band C) was not detected by ^{32}P labeling, indicating that it represents unphosphorylated pRB. This was present at a similar low level in both the wild-type and *p21-/-* irradiated cells. The remaining bands co-migrated with the ^{32}P-labeled band A and band B. Comparison of the ^{32}P and immunoblotting signals for bands A and B suggested that band A corresponds to the hyperphosphorylated form of pRB whereas band B results from the partial phosphorylation of the pRB protein. Significantly, whereas the protein levels of partially phosphorylated pRB (band B) were similar in the wild-type and mutant irradiated cells, the fully phosphorylated form of pRB was significantly reduced in wild-type cells. We therefore conclude that γ-irradiation blocks the conversion of partially- to hyper-phosphorylated pRB in a p21-dependent manner.

Site-specific differences in pRB phosphorylation in wild-type and *p21-/-* irradiated cells

The difference between the partially- and hyper-phosphorylated forms of pRB could be due to differences in either the specific sites of phosphorylation or the extent to which pRB phosphorylated. To distinguish between these two possibilities, we compared the two dimensional tryptic phosphopeptide maps of pRB species isolated from either wild-type (predominantly band B) or *p21-/-* (band A and B) irradiated cells. In wild-type irradiated cells, we detected six prominent pRB tryptic phosphopeptides (1-6; Fig. 3A). Thus, pRB is phosphorylated at multiple sites in wild-type cells after γ-irradiation. The two-dimensional phosphopeptide map of pRB from *p21-/-* cells was considerably more complex, containing more than 15 major phosphopeptides (1-15; Fig. 3A). Six of these phosphopeptides (1-6) were identical to those detected in the map of pRB from wild-type cells. The remainder (phosphopeptides 7-15) were either greatly under-represented or completely absent from the two dimensional map of pRB derived from wild-type cells (Fig. 3A). The simplest interpretation of these data is that the novel phosphopeptides are derived from the *p21-/-* specific species, band A, and that transition from partially- to hyper-phosphorylated pRB must therefore involve the phosphorylation of a novel set of sites. By extension of this logic, we conclude that the *p21-/-* cells must contain at least two pRB-kinases with different site specificities; one that is similarly active in wild-type and *p21-/-* irradiated cells and results in phosphorylation of a subset of pRB sites (phosphopeptides 1-6) and one that is exclusively active in *p21-/-* irradiated cells and results in the hyper-phosphorylation of pRB through the specific modification of a distinct set of sites (phosphopeptides 7-15).

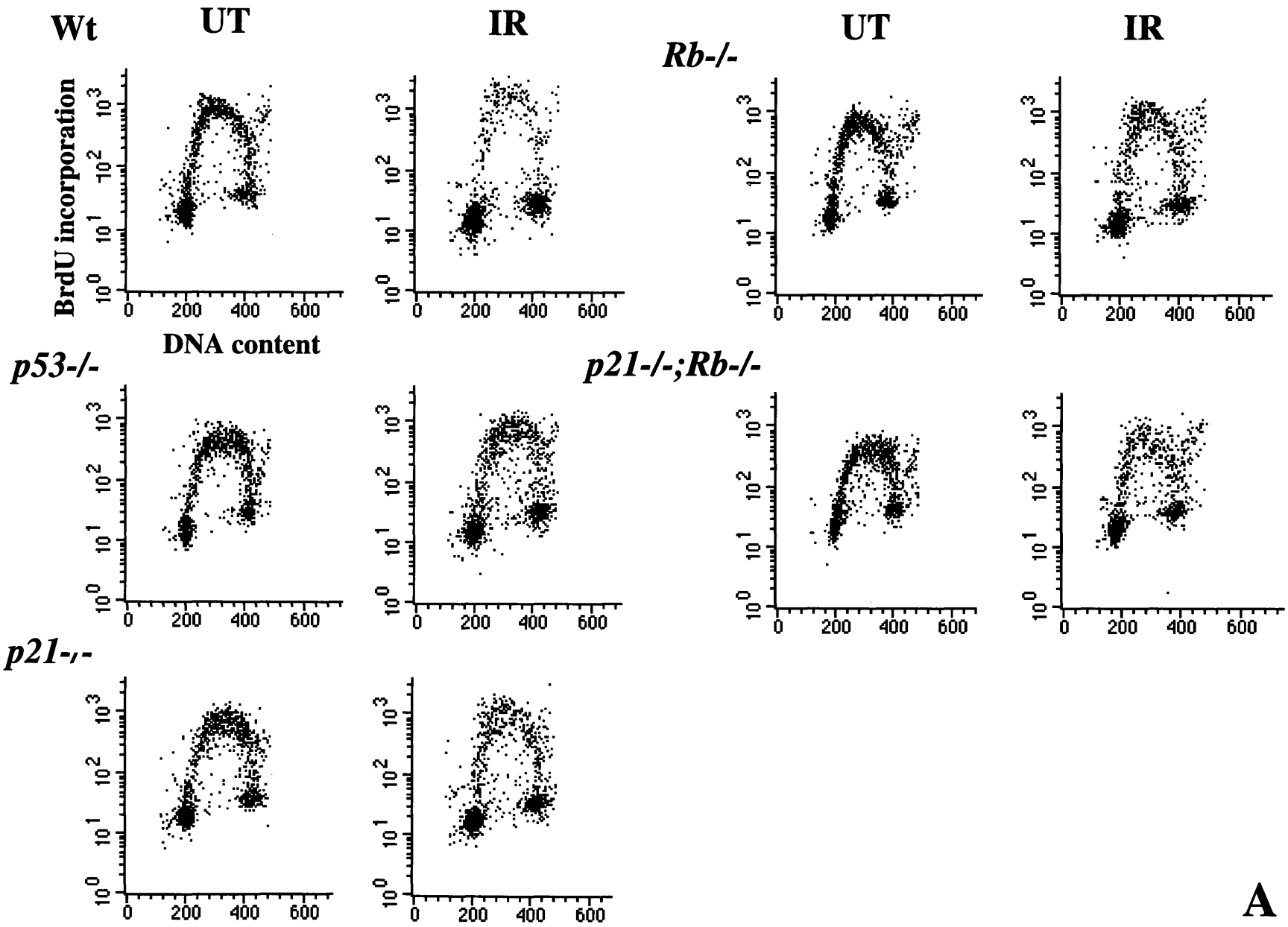
pRB phosphorylation by CDK2 is inhibited in wild-type cells following irradiation

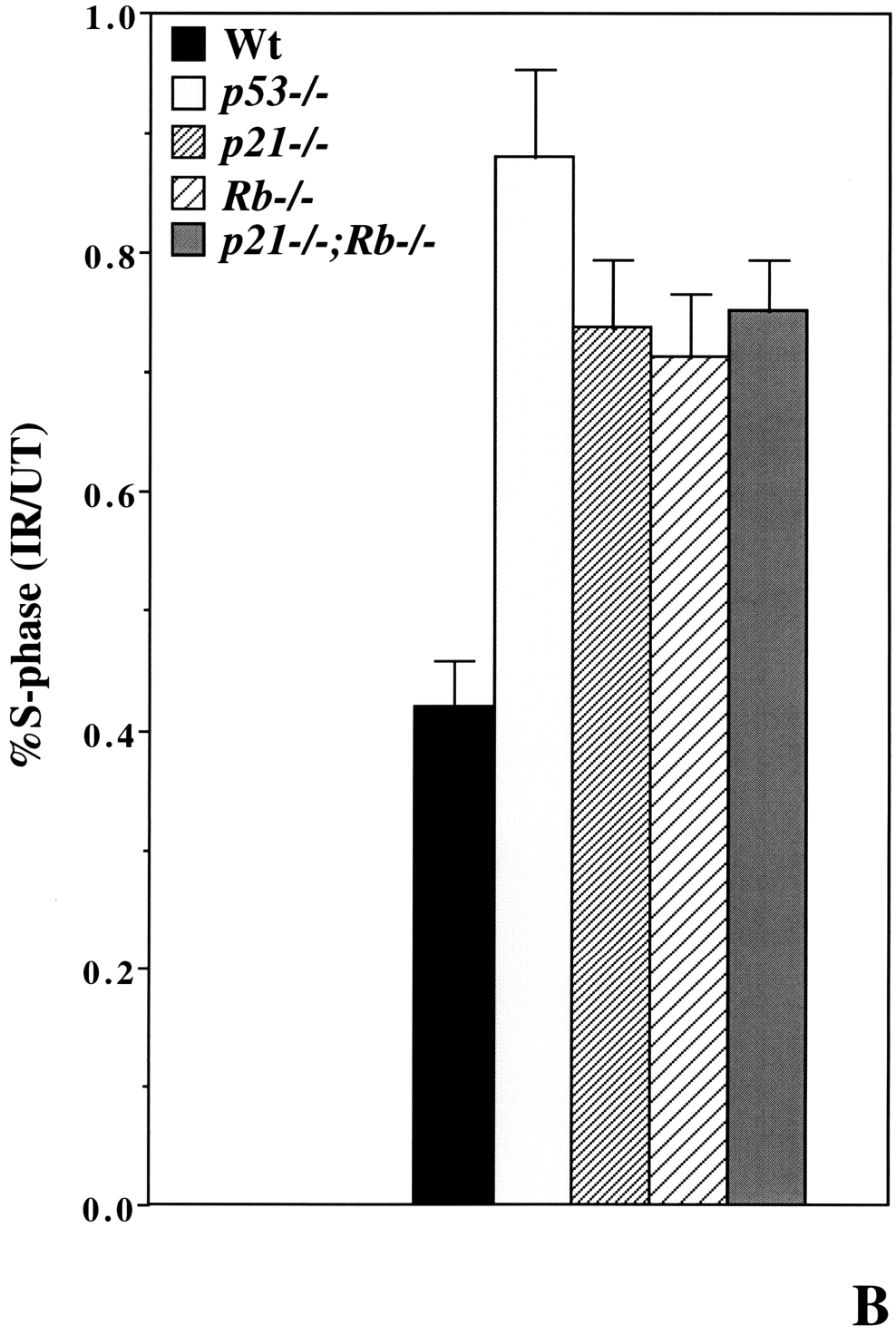
Our kinases assays (Fig. 2A) showed that cdk4 kinase activity was present at similar levels in both wild-type and *p21-/-* irradiated cells but cdk2 activity was only present in *p21-/-*

irradiated cells. This suggested that cdk4 accounts for the partial phosphorylation of pRB (phosphopeptides 1-6), whereas cdk2 is responsible for phosphorylating the second set of sites that switch pRB from the partially- to the hyper-phosphorylated form (phosphopeptides 7-15). To test this hypothesis, we employed a panel of antibodies that specifically recognize individual pRB-phosphopeptides that are preferentially phosphorylated by either cyclinD•cdk4 or cyclinE•cdk2 *in vitro* (Kitagawa et al. 1996). These antibodies were used to screen immunoblots of pRB-immunoprecipitates from either wild-type or *p21-/-* irradiated cells (Figure 3B). CyclinD•cdk4 is known to specifically phosphorylate Ser780 with a 20-60 fold higher efficiency than either Cyclin E•cdk2 or Cyclin A•cdk2 (Kitagawa et al. 1996). Antibodies specific for phospho-Ser780, recognized both the partially and the hyper-phosphorylated form of pRB indicating that the cdk4 kinase contributes to the phosphorylation of both of these species (Fig. 4). In contrast, an antibody directed against a cdk2-specific pRB site (α-phospho-Ser811), recognized the hyper-phosphorylated form of pRB in *p21-/-* irradiated cells but failed to detect the partially phosphorylated pRB species in either wild-type or *p21-/-* irradiated cells (Fig. 3B). Similar results were obtained with an antibody against a second cdk2-specific pRB phosphorylation site, phospho-Thr350 (data not shown). Taken together, these data indicate that the cdk4 kinase is responsible for the partial phosphorylation of pRB *in vivo*, but active cdk2 kinase is required to phosphorylate the additional sites specifically modified in hyper-phosphorylated pRB. Most importantly, our data suggest that the p53 ⇒ p21 pathway arrests cells in response to low doses of γ-irradiation by specifically inhibiting the cdk2 kinase and thereby preventing the transition of pRB from the partially phosphorylated to the hyper-phosphorylated form. Given the requirement of pRB for the integrity of the G_1 arrest, we conclude that partially phosphorylated pRB is responsible for mediating this growth arrest.

Figure 1. DNA damage-induced G_1 arrest response of wild-type, *p53-/-* , *p21-/-*, *Rb-/-*, *p21-/-;Rb-/-*.

(A) Representative two diensional FACS analysis of untreated (UT) and irradiated (IR) samples. Asynchronous cultures were irradiated with a dose of 5.5 Gy and labeled with BrdU for 4 hours beginning 14 hours after irradiation. (B) Histogram showing the S-phase fraction of irradiated versus untreated samples. The mean and standard deviations (error bars) of four independent experiments are shown.


Wt
UT
IR
Rb-/-
UT
IR
BrdU incorporation
DNA content
p53-/-
p21-/-;Rb-/-
p21-/-
A



B

Figure 2. Analysis of cdk4 and cdk2 activities and pRB phosphorylation in wild-type and *p21-/-* cells following irradiation.

(*A*) Cdk4 and cdk2 were immunoprecipitated from wild-type and *p21-/-* cells both untreated and 18 hours after γ-irradiation. Normalized protein extracts were precleared with protein A beads and assayed for kinase activity by incubating the cdk4 and cdk2 immunoprecipitates in the presence of vast excess of both [γ-^{32}P]ATP and substrate (histone H1 or a C-terminal fragment of pRB). Background kinase activity in the absence of cdk immunoprecipitation was minimal (< 10 %; data not shown). (*B*) SDS-PAGE analysis of [^{32}P]pRB from wild-type and *p21-/-* cells 18 hours after irradiation. (*C*) Western blot analysis of [^{32}P]pRB with an α-pRB monoclonal antibody.

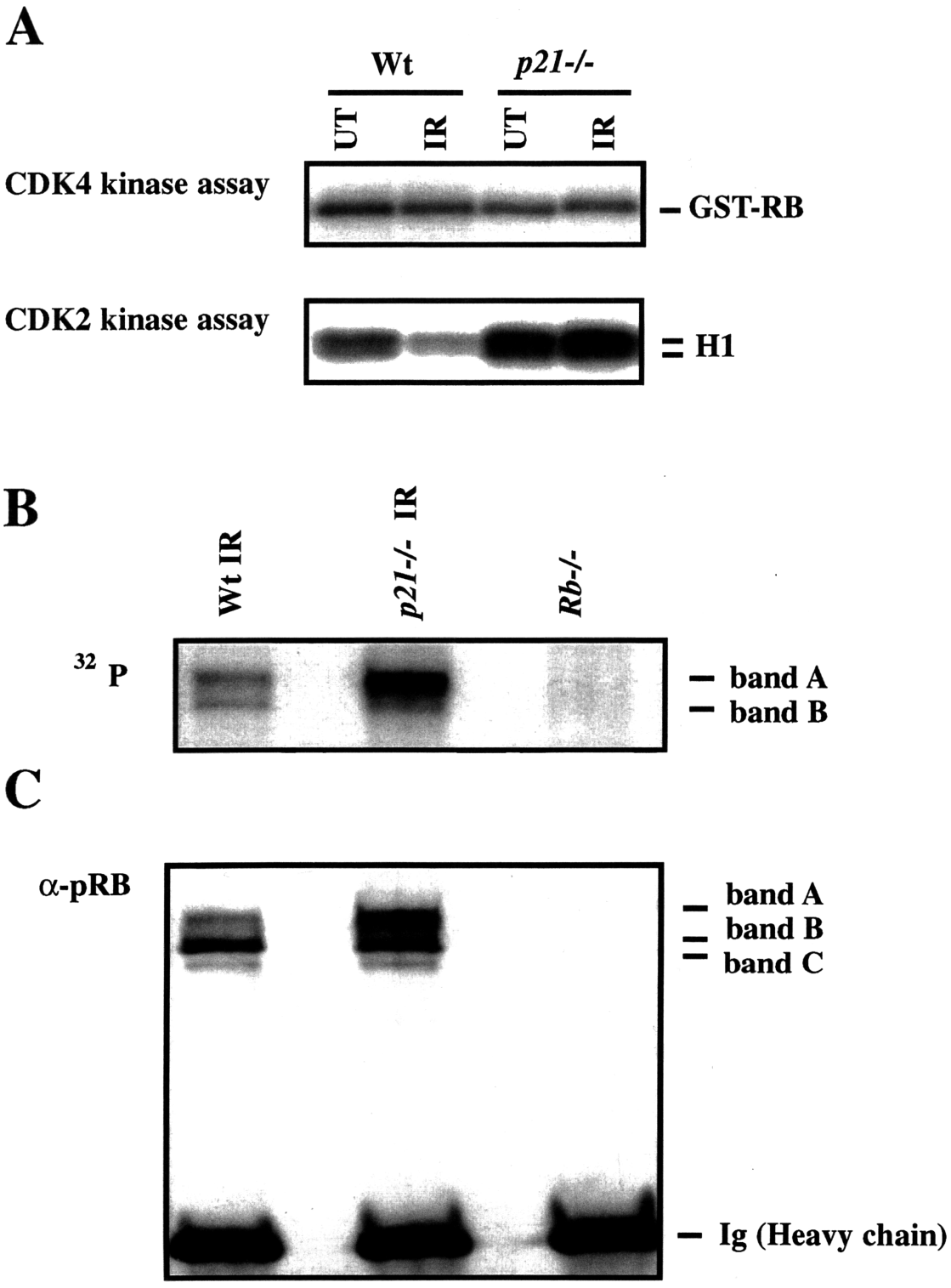
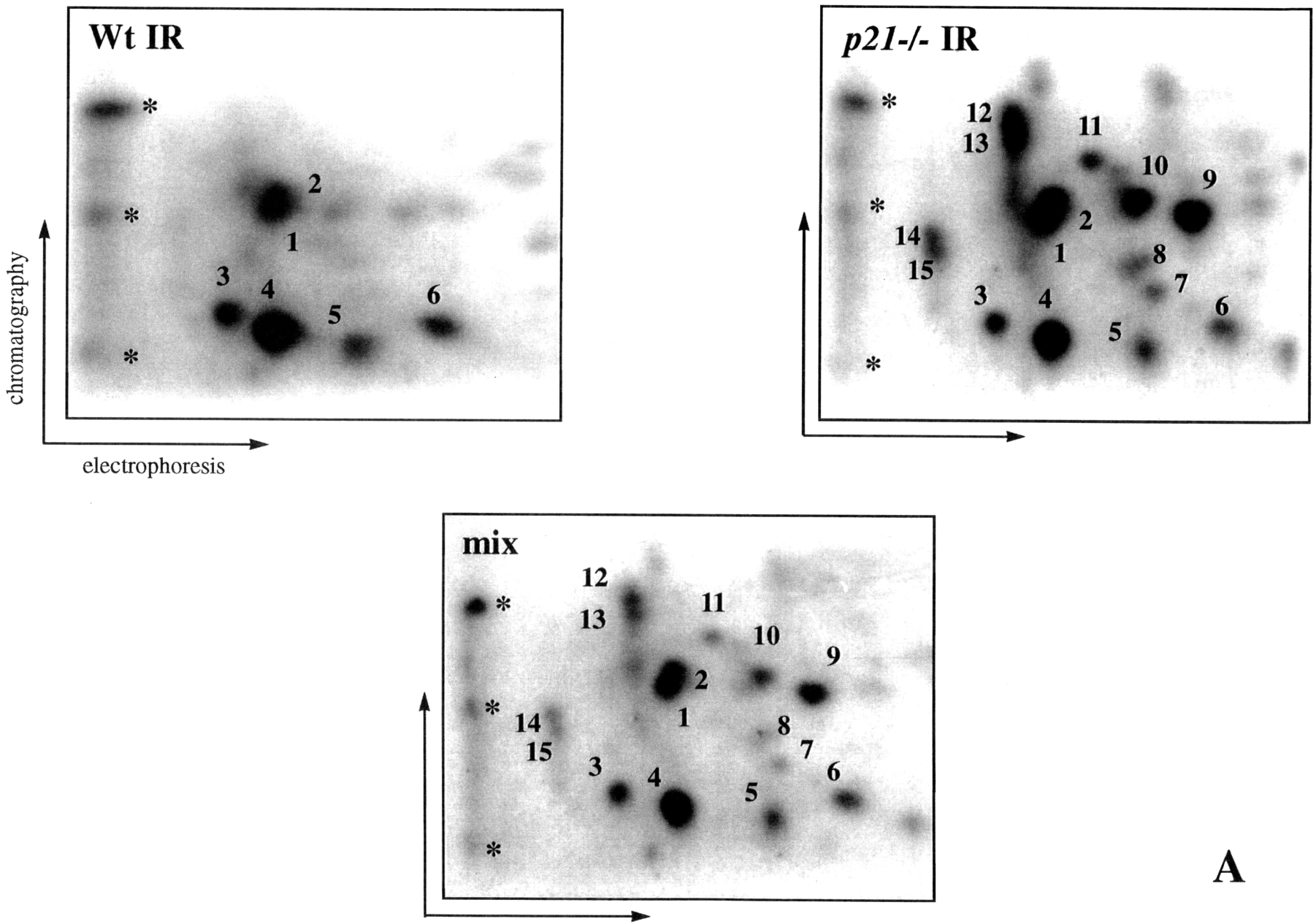
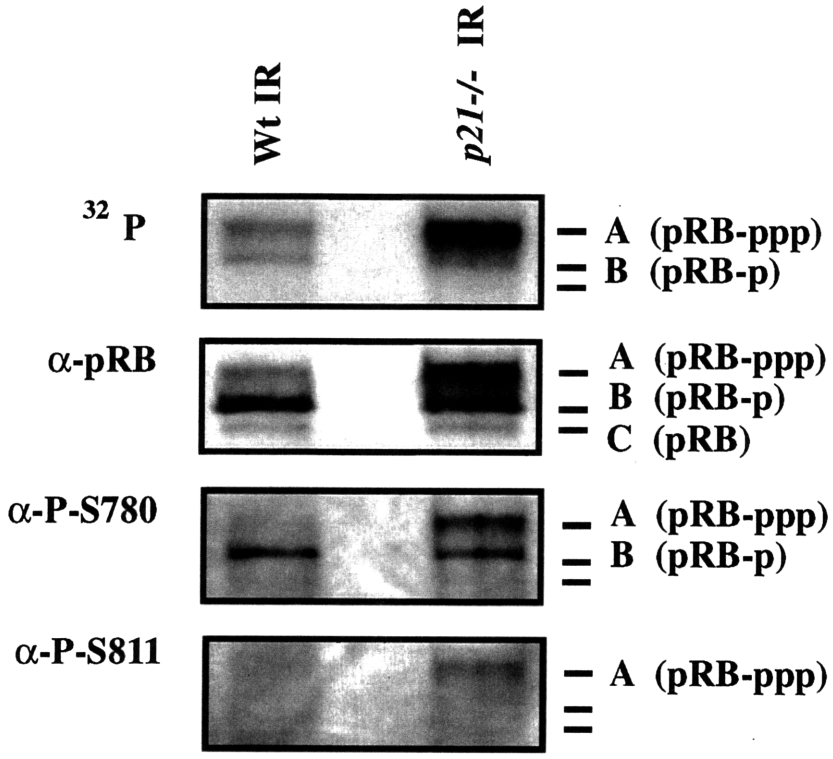

A
Wt
p21-/-
UT
IR
UT
IR
CDK4 kinase assay
GST-RB
CDK2 kinase assay
H1
B
Wt IR
p21-/- IR
Rb-/-
32 P
band A
band B
C
α-pRB
band A
band B
band C
Ig (Heavy chain)

Figure 3. Analysis of pRB phosphorylation from wild-type and *p21-/-* irradiated cells.

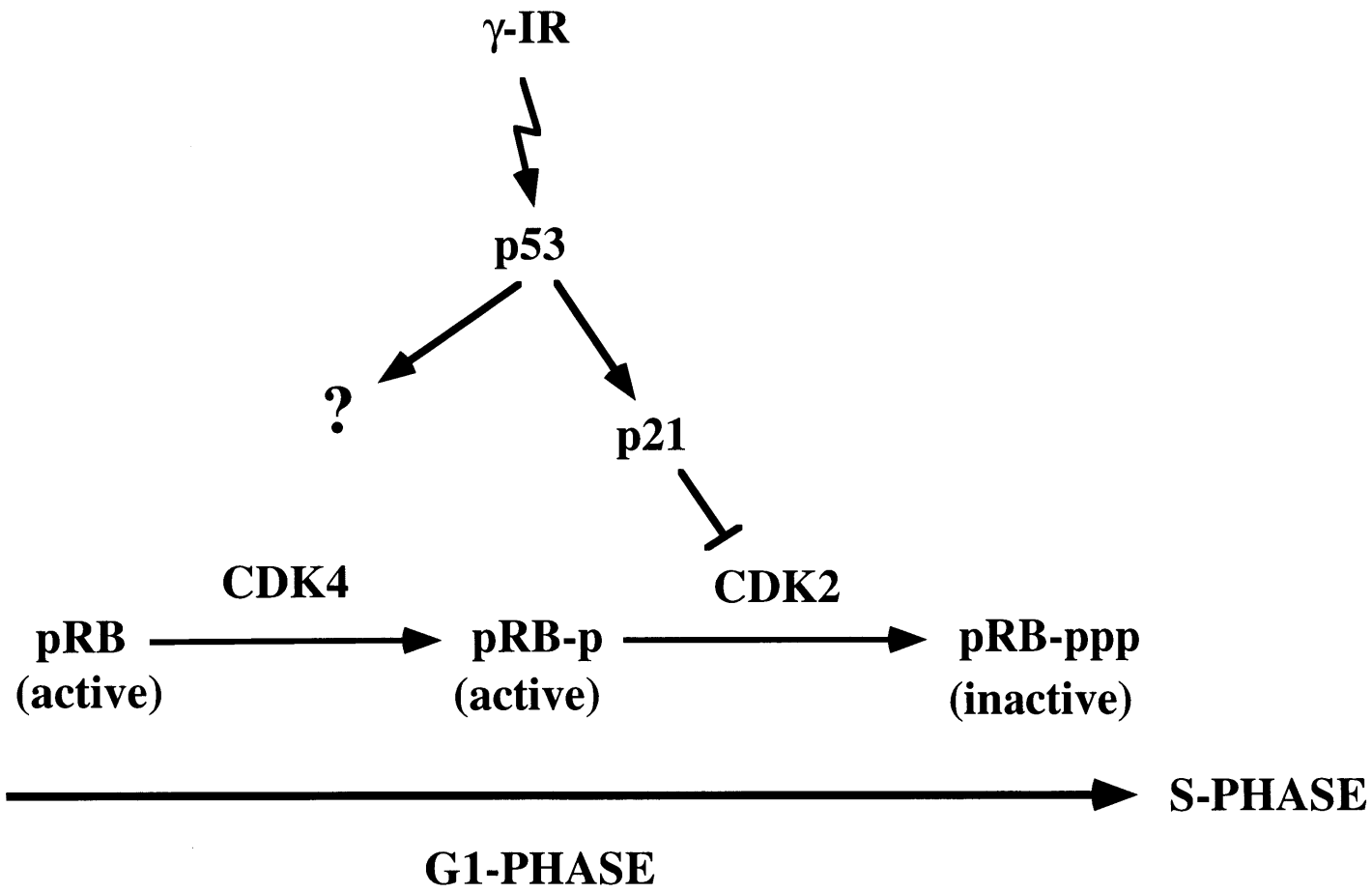
(*A*) pRB tryptic phosphopeptide maps from wild-type and *p21-/-* irradiated cells. A map with pRB phosphopeptides from both wild-type and *p21-/-* cells is also shown. (Phosphopeptides marked with * are not derived from pRB as they were also present in maps from *Rb-/-* cells.) (*B*) Western blot analysis of [^{32}P]pRB with: α-pRB, and phosphopeptide specific antibodies α-P-S780 (cdk4 site) and α-P-S811 (cdk2 site).


Wt IR
p21-/- IR
mix
chromatography
electrophoresis
A


Wt IR
p21-/- IR
32 P
A (pRB-ppp)
B (pRB-p)
α-pRB
A (pRB-ppp)
B (pRB-p)
C (pRB)
α-P-S780
A (pRB-ppp)
B (pRB-p)
α-P-S811
A (pRB-ppp)

B

Figure 4. Model of DNA damage induced G_1-arrest.


γ-IR
p53
?
p21
CDK4
CDK2
pRB
(active)
pRB-p
(active)
pRB-ppp
(inactive)
S-PHASE
G1-PHASE

DISCUSSION

The ability of cells to arrest in G_1 in response to DNA damage is dependent upon the accumulation of the tumor suppressor, p53 (Kastan et al. 1992). This p53-dependent, G_1 arrest is mediated through the activation of two distinct pathways, one of which requires the cdk inhibitor, p21 (Brugarolas et al. 1995; Deng et al. 1995) . By analyzing primary cell lines lacking specific cell cycle regulators, we have established the precise mechanism by which p21 brings about the G_1 arrest that is observed after treatment with low dose γ-irradiation. Our data indicate that p21 specifically inhibits cdk2, thereby preventing the inactivation of the growth suppressive properties of the pRB tumor suppressor. Several lines of evidence support this conclusion. 1) The induction of p21 results in the specific inhibition of cdk2 but not cdk4 kinase activity. 2) The phosphorylation status of pRB is altered in response to γ-irradiation in a p21-dependent manner. 3) The change in pRB phosphorylation arises from the specific loss of phosphorylation at cdk2- but not cdk4-specific sites. 4) Mutation of the *Rb* gene abrogates the ability of the p53 $\Rightarrow$ p21 pathway to block S-phase entry. Taken together, these data indicate that the p53 $\Rightarrow$ p21 checkpoint pathway co-opts the normal cell cycle regulatory mechanisms to induce the accumulation of the growth suppressive form of pRB. In this manner, pRB plays a critical role in determining whether or not a cell will initiate DNA replication in the presence of DNA damage.

Our observations also provide considerable insight into the role of pRB in normal cell cycle control (see Figure 4). Recent studies have shown that pRB is phosphorylated in a two step process during the normal cell cycle (Kitagawa et al. 1996; Ezhevsky et al. 1997; Lundberg and Weinberg 1998). CyclinD•cdk4/6, specifically phosphorylates pRB at a subset of its phosphorylation sites. However, complete phosphorylation of pRB requires cyclinE•cdk2 to specifically target the remaining phosphorylation sites. In each case,

inhibition of cdk2 resulted in a G_1 block that correlates with the inhibition of the second step of pRB phosphorylation (Ezhevsky et al. 1997; Lundberg and Weinberg 1998). Unfortunately, in these cell systems, it is impossible to establish whether the accumulation of partially phosphorylated pRB is responsible for the observed G_1 block. Our current data are entirely consistent with the notion that cyclinD•cdk4/6 and cyclinE•cdk2 mediate the sequential phosphorylation of pRB through the phosphorylation of distinct subsets of the pRB phosphorylation sites. Moreover, using genetically defined cells we directly addressed the relationship between the phosphorylation status of pRB and its ability to block cell cycle progression. Our data indicate that the p53 $\Rightarrow$ p21 checkpoint pathway is able to impose a G_1 block by specifically inhibiting cdk2 kinase activity and thereby the second step of pRB phosphorylation. Although cdk2 inhibition may affect the phosphorylation of other cdk2 targets, our data clearly indicate that the imposition of the G_1 arrest is dependent upon the presence of functional pRB. Based on these findings, we can conclude that partially phosphorylated pRB retains the ability to block cell cycle progression. By extension of this logic, cyclinD•cdk4-dependent phosphorylation is insufficient to inactivate the growth suppressive properties of pRB. Although these conclusions were derived from the analysis of the DNA damage response, it seems highly likely that the same mechanisms will control the timing of S-phase entry in the normal cell cycle.

This model raises clear questions about the role of cyclinD•cdk4 in the regulation of pRB. It is possible that this kinase affects a pRB function that is unrelated to the control of cell cycle entry. However, there is extensive data to suggest that cyclinD•cdk4 plays a critical role in over-riding the growth suppressive properties of pRB (Hinds et al. 1992; Matsushime et al. 1994; Lukas et al. 1995a; Lukas et al. 1995b). Alternatively, the inactivation of pRB by cyclinE•cdk2 may be entirely dependent upon the prior phosphorylation of pRB by cyclinD•cdk4. This mechanism would provide two distinct

points at which extracellular signals and/or checkpoint pathways could influence the state of pRB phosphorylation and therefore the cell division process. Significantly, very high doses of γ-irradiation can result in the inhibition of both cdk2 and cdk4 kinase activity (Terada et al. 1995). Together with our data, this observation suggests that the DNA damage checkpoint can block pRB phosphorylation by specifically inhibiting either one (cyclinE•cdk2) or both (cyclinD•cdk4 and cyclinE•cdk2) of the pRB kinases depending on the severity of the DNA damage. The mechanism of inactivation of cyclinD•cdk4 in this response has yet to be established. However, it is now clear that the modulation of the site-specific phosphorylation state of pRB is a critical control point in both normal cell cycle regulation and the DNA damage checkpoint.

The ability of checkpoint pathways to interface with the cell cycle control machinery is emerging as a common regulatory mechanism in eukaryotes (for review, see Elledge 1998). In *S. pombe*, DNA damage results in a G_2 cell cycle arrest. In this case, γ-irradiation activates a protein kinase, Chk1p, that induces arrest by specifically inhibiting Cdc25p, a phosphatase that is essential for the activation of the cdk, Cdc2p (Furnari et al. 1997). A combination of biochemical studies and the cell cycle effects of exogenously expressed, mutant *cdc25* strongly suggest that this G_2 checkpoint pathway also exists in mammalian cells (Peng et al. 1997; Sanchez et al. 1997). Budding and fission yeast can also arrest in mitosis in response to defects in the mitotic spindle (Hwang et al. 1998). This spindle assembly checkpoint induces a signalling cascade that results in the inactivation of Cdc20p, an essential activator of the APC (Anaphase Promoting Complex) - dependent proteolysis machinery that triggers the metaphase-anaphase transition (Hwang et al. 1998; Kim et al. 1998). As with the Chk1 pathway, the components of both the spindle checkpoint and the APC-dependent proteolysis machinery are well conserved in mammalian cells. In all of these studies, the elucidation of these checkpoint pathways has been largely dependent upon the ability to conduct genetic analyses. The existence of

mouse strains deficient for cell cycle regulators has enabled us to conduct genetic analysis in mammalian cells. Using these cells we have established the precise mechanism of action of a checkpoint pathway that is not conserved in yeast. This approach is likely to become increasingly important to dissect the molecular mechanisms responsible for mediating mammalian checkpoint pathways.

EXPERIMENTAL PROCEDURES

Irradiation and cell cycle analysis

Sparse cultures of MEFs were irradiated with 5.5 Gy using a γ-cell irradiator with a *Cs* source. Untreated and irradiated cultures were harvested for cell cycle analysis 18 hours after γ-irradiation. Cell cycle analysis were performed as described (Brugarolas et al. 1995).

In vitro kinase assays

Cdk2 and cdk4 *in vitro* kinase assays were performed as described (Brugarolas et al. 1998). Briefly, cell lysates were precleared with equilibrated protein A beads (Pierce) and incubated with anti-cdk4 (Santa Cruz, C-22) or anti-cdk2 antibody (kindly provided by G. J. Hannon, CSHL, CSH, NY) for 4 hours. Immune complexes were precipitated with protein A beads (Pierce) and incubated in the kinase buffer containing 4 mM ATP, 20 μCi [γ-^{32}P] ATP (NEN-DuPont) and 6 μg of GST-RB (GST fusion with amino acids 792-928 from the C-terminus of pRB) or 2 μg of histone H1 (Sigma), for 30-60 minutes at 30^{o}C. Quantitation was performed by phophorimager analysis.

^{32}P-labeling and pRB immunoprecipitation

Subconfluent MEFs cultures were labeled with 5 mCi per ml HCl free ^{32}P-orthophosphate (NEN-DuPont) in DME supplemented with 5% dialyzed fetal bovine serum. Labeling proceeded for 4 hours starting 14 hours after γ-irradiation. Protein extracts were prepared as described (Brugarolas et al. 1998), normalized for ^{32}P incorporation and used for pRB immunoprecipitation using monoclonal antibodies XZ104 (Hu et al. 1991), XZ 133 (Hu et al. 1991) and 21C9 (Whyte et al. 1988). pRB was then resolved in a 6% polyacrylamide SDS gel, blotted onto Immobilon-P (Millipore) and

visualized by autoradiography.

Immunoblotting

Membranes were blocked in TBS-T (10 mM Tris (pH 7.5), 150 mM NaCl, 0.03% Tween-20) containing 5% non-fat dry milk. pRB was detected using mouse monoclonal G3-245 (Pharmingen) at a dilution 1:175 and a three step protocol using a rabbit anti-mouse secondary antibody and an anti-rabbit tertiary antibody conjugated to HRP. Phosphoserine 780 and 811 (Kitagawa et al. 1996) were detected using rabbit polyclonal antibodies at 1:300 and 1:100 dilution respectively and a secondary anti-rabbit antibody conjugated to HRP. Detection was performed by enhanced chemiluminescence.

Two-dimensional phosphopeptide mapping

[^{32}P]pRB was immunoprecipitated, blotted and visualized as described above. Both band A and band B were excised and subjected to two dimensional tryptic phospho-peptide mapping as described (Dynlacht et al. 1997).

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Chapter 5

Overview & Future Directions

Analysis of the role of p21 in differentiation

To study the function of the CKI p21 *in vivo* we initially generated chimeric mice composed of wild-type and *p21*-deficient cells. These chimeras were derived from wild-type blastocysts (C57BL/6 genetic background) that were injected with ES cells (129/Sv genetic background) harboring a disruption of the *p21* gene. The analysis of chimeras is extremely sensitive to phenotypic variation allowing the direct comparison of mutant and wild-type cells in the same tissue which is not confounded by mouse to mouse variation. We focused our study of *p21-/-* chimeras to the intestine for several reasons. First, we were interested in analyzing the role of p21 in differentiation and the gut epithelium represents a well characterized system. Second, *p21* is expressed at very high levels in the intestinal epithelium; the intestine is perhaps the tissue that expresses the highest levels of *p21* in the mouse (Macleod et al., 1995). Third, there are readily available markers that distinguish intestinal epithelial cells in a C57BL/6 genetic background (in this case, wild-type cells) from cells in a 129/Sv genetic background (in this case, *p21-/-* cells).

Intestinal crypts and villi composed of *p21*-deficient cells were indistinguishable from wild-type crypts and villi (Brugarolas et al., 1995). *p21-/-* cells were able to differentiate into the four principal intestinal epithelial lineages, and terminally withdraw from the cell cycle. In addition, the rates of migration of *p21-/-* and wild-type epithelial cells across the crypt-villus axis were also the same. This analysis indicated that loss of *p21* did not affect intestinal epithelial differentiation. However, the analysis of the role of p21 in differentiation in other tissues in the chimeras was limited by the lack of tissue specific markers that distinguished mutant and wild-type cells. To overcome this problem we generated a *p21*-deficient mouse strain (Brugarolas et al., 1995).

p21-/- mice developed normally and were born at the expected Mendelian ratios. These mice did not exhibit any overt phenotype and were not predisposed to tumors,

indicating that p21 is not a tumor suppressor protein. This is consistent with the lack of *p21* mutations in human tumors (Shiohara et al., 1994). In addition, histological analysis of multiple tissues from these mice did not reveal any abnormalities. Thus, unlike the other CIP/KIP inhibitors p27 (Kiyokawa et al., 1996; Fero et al., 1996; Nakayama et al., 1996) and p57 (Yan et al., 1997; Zhang et al., 1997), p21 is not required for tumor suppression or differentiation in the mouse (Brugarolas et al., 1995; Deng et al., 1995). These data do not indicate however, that p21 is not involved in differention, but support the idea that p21 is not essential, which could be explained by redundancy or functional compensation by other CIP/KIP inhibitors.

To address whether other CIP/KIP inhibitors were compensating for *p21* loss, we decided to generate mice deficient for multiple CIP/KIPs. To this end, we have generated mice deficient for both p21 and a p27. Preliminary characterization of these mice has revealed some synergistic effects of these mutations. *p21-/-;p27-/-* mice, in contrast to single mutant mice, exhibit a decreased viability; at three weeks of age the ratio of double mutant mice is half of the expected number based on Mendelian genetics (J.B. and T.J. unpublished data). In addition, loss of *p21* seems to accelerate tumor development; double mutant mice develop the same tumors as *p27-/-* mice but at an earlier stage (J.B. and T.J. unpublished data). This phenotype is reminiscent of the effects of *p21* loss in *Rb+/-* mice (Brugarolas et al., 1998). Considered together, these results indicate that p21 and p27 may cooperate in tumorigenesis and may perform partially redundant functions in development.

p21 is a regulator of the normal cell cycle

To assess the role of p21 in the regulation of the normal cell cycle, we isolated fibroblasts from *p21-/-* mouse embryos. The comparison of *p21-/-* to wild-type mouse embryo fibroblasts (MEFs), revealed that loss of *p21* results in multiple cell cycle abnormalities.

p21-deficient fibroblasts exhibited a shorter G1-phase of the cell cycle and a reduced G1 cell size (Brugarolas et al., 1998). These phenotypes have been previously described in cells engineered to have increased G1 CDK activity by overexpression of the CDK4 activatory subunit, cyclin D (Quelle et al., 1993; Resnitzky et al., 1994) or the CDK2 activatory subunit, cyclin E (Ohtsubo et al., 1993; Resnitzky et al., 1994). Therefore, we decided to examine CDK4 and CDK2 activities in *p21*-deficient cells. *p21-/-* cells exhibited 2-4 fold higher levels of CDK2 activity than wild-type cells (Brugarolas et al., 1998). These data indicated that p21 acts to restrain CDK2 activity in exponentially growing fibroblasts which is consistent with *in vitro* studies showing that p21 is a very effective inhibitor of cyclin/CDK complexes containing CDK2. The analysis of CDK4 activity in *p21-/-* and wild-type cells showed no significant differences (Brugarolas et al., 1998), indicating that p21 does not act as a CDK4 inhibitor under these experimental conditions. This was an unexpected result based on the observation that p21 inhibits CDK4 complexes *in vitro* with similar efficiency as CDK2 complexes (Harper et al., 1995). Thus, in exponentially growing fibroblasts p21 acts to specifically regulate CDK2 but not CDK4 activity. The upregulation of CDK2 activity in *p21-/-* cells may explain the G1 shortening and the reduced G1 cell size. In addition, our data undermines the idea that p21 is a critical factor for the assembly of active CDK4 and CDK2 complexes *in vivo* (Zhang et al., 1994; Zhang et al., 1994; LaBaer et al., 1997). If this were true, loss of p21 would have resulted in the decreased CDK4 and CDK2 kinase activities.

In light of the cell cycle phenotype of *p21-/-* MEFs it may be surprising that *p21*-deficient mice develop normally. This could indicate that the effects of p21 are limited to the G1-phase of the cell cycle and that they can be compensated for in other stages of the cell cycle. In support of this idea we have found that in spite of the G1 shortening and the reduced G1 cell size, *p21*-deficient cells have a normal proliferation rate and a normal G2 cell size (Brugarolas et al., 1998). These data suggest that the effects of loss of *p21* in G1

are compensated in other phases of the cell cycle and may explain the lack of an effect of a *p21* mutation in the mouse.

Interestingly, a shortening of the G1 phase has also been observed in cells deficient for the CKI, Sic1 (Schwob et al., 1994). Sic1 is an inhibitor of the S-phase CDKs in *S. cerevisiae* (Clb5 (6)/Cdc28) and *sic1-Δ* results in the premature activation of Clb5 (6)/Cdc28 and a consequently, premature S-phase entry. To determine the role of p21 in the regulation of the timing of CDK2 activation we compared the CDK2 kinase activity of wild-type and *p21-/-* cells as they proceeded from G0 into S-phase. In *p21-/-* cells, CDK2 was activated two hours earlier than in wild-type cells (J.B. and T.J. unpublished data) suggesting that p21 acts to prevent premature activation of CDK2 in G1. These data suggest that CKIs are important in the regulation of the timing of activation of CDKs during G1 and that this function may be conserved among species.

Regulation of cell proliferation by extracellular signals

Mitogenic signals are thought to induce proliferation by activating the G1 CDKs CDK4 and CDK2. Thus, it was interesting to study the effects of loss of *p21* in the regulation of the cell cycle in response to extracellular signals. Despite the fact that loss of *p21* resulted in a significant upregulation of CDK2 activity, this had limited effects in the regulation of the cell cycle by environmental signals. *p21-/-* cells required lower concentrations of growth factors for proliferation than normal cells but effectively arrested proliferation at high cell densities and under conditions that did not allow attachment and spreading onto a substratum (Brugarolas et al., 1998). It is possible that loss of *p21* is not sufficient to maintain high levels of CDK2 activity under these conditions and that this may explain the arrest. However, at least in anchorage-independent conditions, *p21*-deficient cells exhibited elevated CDK2 activity compared to wild-type cells (Brugarolas et al., 1998) and yet they

still arrested. This indicated that elevated levels of CDK2 activity are not sufficient for cell proliferation under antimitogenic conditions. This arrest may be mediated through the inhibition of CDK4. To test this idea, we set up to identify a cell type with elevated CDK4 activity that could be tested in these assays.

There is compelling evidence to suggest that the growth promoting effects of CDK4 are mediated through the inactivation of the growth suppressor, the retinoblastoma protein (pRB). Indeed, the effects of loss of *Rb* (Lukas et al., 1995a; Herrera et al., 1996) are comparable to the effects of constitutive activation of CDK4 by ectopic cyclin D1 expression (Quelle et al., 1993; Resnitzky et al., 1994). In addition, whereas inhibition of CDK4 activity by α-cyclin D1 antibodies or INK4 inhibitors is sufficient to stop normal cell proliferation this has no effect on the proliferation of cells lacking *Rb* (Guan et al., 1994; Koh et al., 1995; Lukas et al., 1995a; Lukas et al., 1995b; Medema et al., 1995). Therefore *Rb*-deficient cells are likely to be resistant to antimitogenic signals that arrest proliferation by inhibiting the activity of CDK4. We have analyzed the proliferative response of *Rb-/-* MEFs at high cell densities and in anchorage-independent conditions. Similar to *p21-/-* cells, cells lacking *Rb* efficiently stopped proliferation under these antimitogenic conditions indicating that the arrest was not dependent on the inhibition of CDK4. However, this arrest could be mediated through the inhibition of CDK2 as it has been shown that CDK2 inhibition arrests cells irrespective of the *Rb* status (Ohtsubo et al., 1995). Thus CDK2 inhibition at high cell densities or in anchorage-independent conditions could explain the arrest of *Rb-/-* cells. Together with the experiments with *p21-/-* cells, these data supports a model whereby proliferation is regulated by the coordinated activities of both CDK4 and CDK2 such that inhibition of either kinase blocks cell proliferation.

To test this model, we generated cells deficient for both *Rb* and *p21* (Brugarolas et al., 1998). The initial characterization of these cells revealed that these two mutations had additive effects in G1 regulation; *Rb-/-;p21-/-* cells exhibited a significantly shorter G1-

phase and a reduced G1 cell size compared to either single mutant cells (Brugarolas et al., 1998). We tested double mutant cells for their ability to adjust cell proliferation to environmental conditions. Combined inactivation of *Rb* and *p21* resulted in increased proliferation at concentrations of serum insufficient for normal cell growth (Brugarolas et al., 1998). Most strikingly, *Rb-/-;p21-/-* cells proliferated in anchorage-independent conditions (soft agar) (Brugarolas et al., 1998). This indicated that combined loss of *Rb* and *p21* is sufficient for anchorage-independent growth. This effect could not be attributed to a signaling defect as double mutant cells (similar to wild-type cells) downregulated cyclin D1 and CDK2 activity when placed in soft agar (Brugarolas et al., 1998) indicating that these cells are effectively sensing and responding to the lack of adequate cell-substratum interactions. However, in contrast to wild-type cells, the downregulation of cyclin D1 (the major CDK4 activatory cyclin in fibroblasts) and CDK2 activity was not sufficient to block proliferation in *Rb-/-;p21-/-* cells. Loss of *Rb* had rendered the cell cycle machinery unresponsive to changes in CDK4 activity and loss of p21 prevented complete inhibition of CDK2 activity. Thus, despite the downregulation of CDK2 activity in anchorage-independent conditions, *p21-/-;Rb-/-* cells still maintained levels of CDK2 activity that were sufficient for cell proliferation (Brugarolas et al., 1998). Taken together these data indicate that constitutive activation of the CDK4 pathway through loss of *Rb* and the CDK2 pathway through loss of *p21* is sufficient to drive cell proliferation in the absence of an adequate cell-substratum interaction. In addition, this supports the idea that extracellular signals control cell proliferation by regulating the activity of the G1 CDKs, CDK4 and CDK2, and that constitutive activation of these kinases is sufficient to insulate the proliferative machinery from the cellular environment. This is precisely what characterizes tumor cells; tumor cells proliferate autonomously, irrespective of their environment (Sherr, 1996). Thus, it is not surprising that a large number of mutations found in tumors affect regulatory components of the CDK4 and CDK2 pathways (Hirama

and Koeffler, 1995). These two pathways however, are not altered in tumors to the same extent, regulatory proteins of the CDK4 pathway appear mutated in tumors more frequently than CDK2 regulators (Hirama and Koeffler, 1995). This might reflect the relative importance of these two pathways for proliferation (Sherr, 1996). CDK4 is the first CDK to be activated in response to proliferative signals and its activation may contribute to the activation of CDK2. Thus, over-stimulation of the CDK4 pathway may suffice to upregulate the CDK2 pathway to a degree that is sufficient to drive cell proliferation.

If constitutive activation of the CDK4 and CDK2 pathways is sufficient to confer proliferation autonomy, *Rb-/-;p21-/-* cells, which do not require CDK4 activity for proliferation and contain higher levels of CDK2 activity than normal cells, would be expected to be tumorigenic. This idea was tested by analyzing the capacity of *Rb-/-;p21-/-* MEFs to form tumors in immunocompromised nude mice (Brugarolas et al., 1998). Whereas *p53-/-* cells transformed with both the viral oncogene *E1A* and an activated allele of *ras* readily formed tumors in nude mice, *Rb-/-;p21-/-* cells did not, indicating that loss of *Rb* and *p21* is not sufficient for tumorigenesis (Brugarolas et al., 1998). These data would suggest that activation of the CDK4 and CDK2 pathways is not sufficient for oncogenic transformation. However, it is possible that these pathways could be downregulated despite absence of pRB and p21. In support of this idea, we have found that CDK2 activity is effectively inhibited in *Rb-/-;p21-/-* cells at high cell densities (Brugarolas et al., 1998). This result, may also explain the inability of *Rb-/-;p21-/-* cells to overcome contact inhibition of growth. CDK2 inhibition at high cell densities may be mediated by p27, which is upregulated several fold under these conditions (Polyak et al., 1994; Brugarolas et al., 1998) These data illustrate the fact that there are multiple regulatory mechanisms that control the activities of CDK4 and CDK2 and it also underscores the importance of proper regulation of these pathways for normal cell

proliferation.

p21 and pRB in tumorigenesis

In spite of the fact that *p21* mutation is not sufficient to constitutively activate the CDK2 pathway, loss of *p21* cooperated with an *Rb* mutation in tumorigenesis (Brugarolas et al., 1998). It is well established that pRB is a tumor suppressor and mice lacking one allele of *Rb* are predisposed to tumors, including pituitary adenocarcinomas, C-cell tumors (tumors of calcitonin secreting cells) and pheochromocytomas (tumors of the adrenomedulla) (Jacks et al., 1992; Lee et al., 1992; Williams et al., 1994). Significantly, loss of *p21* accelerated tumor development in *Rb+/-* mice (Brugarolas et al., 1998). This occurred without a detectable change in tumor type and resulted in the decreased survival of *p21-/-;Rb+/-* mice by about three months compared to *Rb+/-* mice. Thus, while loss of *p21* does not predispose to tumors it can enhance tumor development.

Future experiments to address the role of CDK4 and CDK2 pathways in differentiation

Proper regulation of the cell cycle is not only necessary to prevent tumor formation but it is also required for cell differentiation. Terminal differentiation demands withdrawal from the cell cycle which typically occurs during the G1 phase as indicated by the fact that most differentiated cells contain a 2n DNA content. Therefore, in this case as well, proper regulation of the CDK4 and CDK2 pathways is likely to be of uttermost importance. Consistent with this idea, *Rb-/-* cells are impaired in their capacity to terminally differentiate into certain lineages such as skeletal muscle or lens fibres (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Morgenbesser et al., 1994; Novitch et al., 1996; Zacksenhaus et al.,

1996). Loss of *Rb* in these cell types leads to reduced expression of differentiation markers and aberrant proliferation. Differentiation defects associated with loss of *Rb* are, however, limited to a few lineages and *Rb-/-* cells have been shown to successfully contribute to most if not all lineages in the adult mouse in a chimeric model system (Williams et al., 1994). Thus, *Rb-/-* cells albeit somewhat impaired in some contexts, can still arrest in response to the majority of differentiation stimuli. This arrest could obviously be mediated through the inhibition of CDK2, possibly through p21. Consistent with this, *p21* is upregulated several fold in *Rb*-deficient cells (Brugarolas et al., 1998) and there is vast evidence supporting a role for p21 in terminal differentiation. For instance, it has been shown that the pattern of expression of *p21* in the developing mouse correlates with terminal differentiation in many cell types (Parker et al., 1995). To test this idea we performed histological analysis on *Rb-/-;p21-/-* embryos (J.B. and T.J. unpublished data). These embryos, died in gestation around the same time as *Rb-/-* embryos (~13.5 dpc, days post-coitus) and were phenotypically very similar to *Rb-/-* embryos showing increased cell proliferation and death of neural tissues, anemia, and generalized edema (J.B. and T.J. unpublished data). This would suggest that loss of *p21* has no effects on *Rb-/-* cell differentiation, however, at this stage of development there are very few tissues that have undergone terminal differentiation. At 13.5 dpc some neurons are terminally differentiated but this process is likely not to require p21, as *p21* is not expressed in the mouse brain during development (Parker et al., 1995). To study the role of p21 and pRB in differentiation in the adult mouse we are in the process of generating *Rb-/-;p21-/-* ES cells carrying the *LacZ* transgene (as a marker). At this stage, we have generated three independent male cell lines that are *p21-/-;Rb+/-*, LacZ positive. These cells were derived *de novo* from embryos generated by crossing *p21-/-;Rb+/-* mice carrying the LacZ transgene and have been tested for their totipotentiality in chimeras. All three cell lines gave rise to high contribution chimeras and went germ line indicating successful derivation

(J.B. and T.J. unpublished data). These cells will be used to generate *p21-/-;Rb-/-* LacZ positive ES cells by retargeting of the wild-type Rb allele in tissue culture. The study of these ES cells in the chimeras promises to be very exciting.

The role of p21 in p53-mediated apoptosis and G1 arrest

Abnormal proliferation due to inappropriate activation of G1 CDK pathways in cells programmed to terminally differentiate activates checkpoint mechanisms that restrain cell proliferation. The abnormal proliferation of *Rb-/-* cells in the lens, for instance, results in cell death (Morgenbesser et al., 1994). Similarly, cell death is also observed in inappropriately proliferating neurons in CNS of the *Rb-/-* embryo (Jacks et al., 1992; Lee et al., 1992; Clarke et al., 1995). Interestingly, this death can be suppressed in a *p53* mutant background (Morgenbesser et al., 1994; Clarke et al., 1995; Macleod et al., 1996). Thus, failure to withdraw from the cell cycle in cells programmed to terminally differentiate results in the activation of the p53 checkpoint pathway and cell death. It is very interesting that p53 is involved in checking undue proliferation associated with failure to terminally differentiate. The p53 checkpoint pathway is known to play a major role in preventing tumor formation. The fact that the majority of tumors in humans have mutated this pathway suggest that disruption of this checkpoint is extremely important for tumor development. Consistent with this, loss of *p53* in *Rb+/-* mice, results in the appearance of novel tumor types such as islet cell tumors or bronchial hyperplasias (Williams et al., 1994; Clarke et al., 1995); tumor formation in these cell types in the *Rb+/-* mice is likely to have been prevented by the p53 checkpoint which would eliminate abnormally proliferating cells in the early stages of tumorigenesis. This phenomenon has also been documented in other systems. Directed expression to the choroid plexus of a truncated form of the viral oncoprotein Large T Antigen (T121), which is still capable of inactivating the pRB family

of growth suppressors (but not p53), in transgenic mice results in abnormal cell proliferation and apoptosis (Symonds et al., 1994). Significantly, p53 elimination in this context abolishes cell death and results in early tumor development (Symonds et al., 1994). Furthermore, transgenic mice that are heterozygous for *p53* exhibit two types of choroid plexus lesions, benign hyperplastic lesions with high levels of apoptosis and malignant tumors with no apoptosis (Symonds et al., 1994). Genetic analysis of the *p53* status in these lesions showed that whereas the hyperplastic lesions contained a wild-type *p53* allele, this was lost in malignant tumors (Symonds et al., 1994). These data argue very strongly that the p53 checkpoint pathway is extremely important to prevent inappropriate cell proliferation. Thus, in addition to constitutive activation of the CDK4 and CDK2 pathways, tumor formation is likely to require inactivation of this checkpoint mechanism. Considered together these data indicate that the p53 checkpoint pathway is important in both tumor suppression as well as in preventing abnormal proliferation of cells programmed to terminally differentiate.

Interestingly, *p21* which is a p53 responsive gene (El-Deiry, 1993), is upregulated in cells undergoing p53-mediated cell death (El-Deiry et al., 1994), suggesting that p21 may be involved in apoptosis. p53-mediated apoptosis can be induced in response to a variety of conditions including DNA damage (Clarke et al., 1993; Lowe et al., 1993; Clarke et al., 1994; Merritt et al., 1994). DNA damage induced by γ-irradiation (IR) in the mouse results in p53-mediated cell death in multiple cell types including stem cells from the intestinal crypts (Clarke et al., 1994; Merritt et al., 1994). We sought to determine the role of p21 in this process by analyzing the irradiation response of *p21*-deficient stem cells in the intestinal crypts of chimeric mice. *p21-/-* intestinal stem cells underwent apoptosis in response to IR (Brugarolas et al., 1995). Furthermore, the number of apoptotic cells in *p21-/-* and wild-type crypts was indistinguishable, indicating that loss of *p21* does not affect p53-mediated apoptosis induced by DNA damage (Brugarolas et al., 1995). p53-

mediated cell death can also be induced by the expression of viral oncoproteins like the adenoviral protein E1A (Rao et al., 1992; Lowe and Ruley, 1993) or the truncated form of LargeT Antigen, T121 (Symonds et al., 1994). We have tested the role of p21 in p53-mediated apoptosis induced by both the viral oncogene *E1A* in fibroblasts in tissue culture as well as by *T121* in the transgenic model system described above. Our data indicate that p21 is also not necessary for p53-mediated cell death induced by the expression of viral oncoproteins (Attardi et al., 1996; C.Y. and J.B. data not shown).

Activation of the p53 checkpoint pathway in normal fibroblasts does not lead to apoptosis but to a cell cycle arrest which occurs in the G1-phase. We determined the role of p21 in this arrest by analyzing the response of *p21*-deficient fibroblasts to γ-irradiation which induces a p53-mediated G1 arrest. *p21-/-* fibroblasts were defective in the G1 arrest following IR indicating that p21 is an important target of p53 in this response (Brugarolas et al., 1995; Deng et al., 1995). Loss of *p21* however, did not affect this arrest to the same extent as loss of *p53* indicating that p21 is not the only target of p53 in this response (Brugarolas et al., 1995; Deng et al., 1995). To characterize the p21-independent p53 pathway we have generated *p21-/-;p53-/-* fibroblasts that have been transduced with a temperature sensitive allele of *p53*. Activation of p53 at the restrictive temperature in this cell type may result in a cell cycle arrest which would be independent of p21. If this were the case, this system could be utilized to identify p53 targets other than p21 involved in G1 arrest.

Despite the fact that p21 is only one component of the p53-mediated DNA damage response, p21 plays a critical role in this process as suggested by the observation that *p21-/-* mice (Barlow et al., 1997), like *p53-/-* mice (Kemp et al., 1994; Lee et al., 1994), are radiosensitive.

We have sought to determine the mechanism of growth arrest by p21 (J.B., K.M., Y.T., T.J., and J.A.L. submitted). To this end we have analyzed the activities of the G1

CDKs in response to γ-irradiation and we have found that whereas CDK4 activity does not change following irradiation, CDK2 is significantly downregulated. To establish whether CDK2 inhibition is mediated by p21 we analyzed CDK2 kinase activity in *p21-/-* cells following irradiation. CDK2 activity was not downregulated in *p21-/-* IR cells indicating that p21 is necessary for the DNA damage-induced inhibition of CDK2. Both G1 CDKs, CDK4 and CDK2, have been implicated in the phosphorylation of the retinoblastoma protein, pRB. We have assessed whether the inactivation of CDK2 following irradiation had any effects on pRB phosphorylation and we have found that irradiated fibroblasts accumulate a species of pRB that is partially phosphorylated, which has been phosphorylated by CDK4 but not CDK2. The accumulation of partially phosphorylated pRB following irradiation was dependent on CDK2 inhibition and did not occur in p21-deficient irradiated cells. To establish whether this species of pRB is contributing to the G1 arrest we determined the irradiation response of *Rb*-deficient fibroblasts. *Rb-/-* MEFs were defective in their G1 arrest response to irradiation which indicated that pRB is required for the arrest and that the partially phosphorylated species of pRB is growth suppressive. Significantly, the degree of impairment of the irradiation response of *Rb-/-* cells was very similar to *p21-/-* cells, indicating that p21 and pRB may be in the same arrest pathway. To test this idea we analyzed the irradiation response of *Rb-/-;p21-/-* MEFs. We have found that double mutants have a response to irradiation that is indistinguishable from single mutant cells indicating that p21 and pRB are in the same genetic pathway in the G1 arrest response to DNA damage. Taken together our data indicate that p53 arrests proliferation in response to irradiation by inhibiting CDK2, through the upregulation of p21, and maintaining pRB in a partially phosphorylated growth suppressive state.

The accumulation of partially phosphorylated pRB in response to irradiation is reminiscent of the effects of pheromone treatment on pRB phosphorylation in yeast

transduced with a human *Rb* expression vector (Hatakeyama et al., 1994). In this strain, the transcriptional upregulation of the CKI Far1 in response to pheromone stimulation results in the inhibition of the G1 CDK Cln2/Cdc28, which is the cyclin E/CDK2 homologue, and the accumulation of partially phosphorylated pRB. Thus, both the pheromone pathway as well as the DNA damage response pathway lead to the transcriptional upregulation of a CKI which is necessary for the inhibition of a late G1 CDK and the implementation of an arrest. Interestingly, in both mammalian and yeast cells this results in the accumulation of partially phosphorylated pRB.

Conclusion

Using a genetic approach we have shown that the G1-phase of the cell cycle is regulated by two partially independent pathways, one that is dependent on CDK4 and another that is dependent on CDK2. Mutations in two negative regulators of these pathways, pRB and p21, have additive effects in G1. Furthermore, loss of both p21 and pRB causes proliferation under conditions that normally induce quiescence. *Rb-/-;p21-/-* cells were anchorage-independent for growth and proliferated at concentrations of growth factors insufficient for normal cell proliferation. Our data support a model whereby constitutive activation of the CDK4 and CDK2 pathways is sufficient to drive cell proliferation.

In addition, we have shown that the p53 DNA damage checkpoint pathway acts to arrest cells by co-opting the normal cell cycle machinery. p53 activation in response to γ-irradiation in fibroblasts resulted in the upregulation of p21 and the inhibition of CDK2 which is necessary for the transition from partially phosphorylated pRB (growth suppressive) to fully phosphorylated pRB (inactive). Thus, p53 arrests cells by blocking the normal process of inactivation of the retinoblastoma protein that occurs during G1.

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