Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by $p27^{Kip1}$ and $p21^{Cip1}$

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Summary

$p27^{Kip1}$ and $p21^{Cip1}$ are thought to suppress tumor growth and prevent cell cycle progression by inhibiting Cdk2-cyclin E/A kinases. Since Cdk2 is dispensable for mitotic cell division, we analyzed the activity of these inhibitors in Cdk2-deficient cells. Ectopic expression of $p27^{Kip1}$ or $p21^{Cip1}$ efficiently inhibits cell cycle progression of *Cdk2*$^{-/-}$ fibroblasts. Loss of $p27^{Kip1}$ or $p21^{Cip1}$ confers similar proliferative advantages to *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ cells. Moreover, Cdk2 is dispensable for $p21^{Cip1}$-induced cell cycle arrest after DNA damage. Finally, ablation of Cdk2 in *p27*^{Kip1} null mice does not suppress their phenotypic defects, including development of pituitary tumors. These results indicate that Cdk2 is not an essential target for $p27^{Kip1}$ and $p21^{Cip1}$ in cell cycle inhibition and tumor suppression.

Introduction

Coordinated regulation of cell cycle progression is essential for normal development and homeostasis. Two families of cell cycle inhibitors play vital roles in this process (reviewed in Sherr and Roberts, 1999). The INK4 protein family specifically binds to Cdk4 and Cdk6 to inhibit interaction with their activating subunits, the D type cyclins. On the other hand, the Cip/Kip family of proteins, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, form inactive complexes with Cdk2-cyclin E and Cdk2-cyclin A. $p21^{Cip1}$ and $p27^{Kip1}$ also bind to Cdk4/6-cyclin D complexes but do not interfere with their kinase activity, at least under stochiometric conditions (Blain et al., 1997; LaBaer et al., 1997). Indeed, these cell cycle inhibitors contribute to the formation of stable Cdk4/6-cyclin D complexes during the early phases of the cell cycle (Blain et al., 1997; LaBaer et al., 1997; Cheng et al., 1999). More recently, the possibility that $p21^{Cip1}$ and $p27^{Kip1}$ may have additional roles outside the nucleus is receiving increasing attention (reviewed in Denicourt and Dowdy, 2004).

In spite of their similar mechanism of action, $p21^{Cip1}$ and $p27^{Kip1}$ play distinct biological roles within the cell. $p21^{Cip1}$ is a transcriptional target of p53 and it is believed to be one of the main effectors of p53-mediated cell cycle arrest (reviewed in Nakayama and Nakayama, 1998; Sherr and Roberts, 1999). In contrast, $p27^{Kip1}$ appears to be a primary negative regulator during normal cell proliferation in a variety of cell types (Sherr and Roberts, 1999). Expression of $p27^{Kip1}$ is controlled by the forkhead family of transcription factors, a group of downstream effectors of the PI3Kinase/Akt signal transduction pathway (Collado et al., 2000). Moreover, $p21^{Cip1}$ and $p27^{Kip1}$ differ at their carboxy-terminus, a domain that provides them with specific functions (Nakayama and Nakayama, 1998; Sherr and Roberts, 1999). For instance, $p21^{Cip1}$ uses this domain to bind to the proliferating-cell nuclear antigen (PCNA), a DNA polymerase delta processivity factor, thus preventing DNA replication (Chen et al., 1995; Waga et al., 1994; Li et al., 1994; Luo et al., 1995).

Targeted deletion of $p21^{Cip1}$ in mice does not cause major phenotypic abnormalities. However, $p21^{Cip1}$-deficient mice develop a variety of tumors, albeit with long latencies (Martín-Caballero et al., 2001). Moreover, *p21*$^{Cip1-/-}$ cells are significantly deficient in their ability to arrest in G1 in response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995). Ablation of *p27*^{Kip1} in mice results in hyperplasia leading to generalized organomegaly and increased body size. Moreover, these animals develop retinal dysplasia and pituitary tumors of the intermediate lobe (Nakayama et al., 1996; Kiyokawa et al., 1996; Fero et al., 1996). *p27*^{Kip1} heterozygous mice are more susceptible to radiation- or ENU-induced tumors. None of these tumors loses its wild-type allele, indicating that *p27*^{Kip1}

SIGNIFICANCE

Controlled synthesis and degradation of the cell cycle inhibitor $p27^{Kip1}$ are key events in regulating cell cycle progression. It is generally accepted that $p27^{Kip1}$, and the related $p21^{Cip1}$ inhibitor, block the cell cycle by inhibiting the kinase activity of Cdk2-cyclin E/A complexes. Here, we provide genetic evidence that Cdk2 is dispensable for the inhibitory activity of $p27^{Kip1}$ and $p21^{Cip1}$ during the G1 phase of the cell cycle and for their tumor-suppressing properties. Whereas the precise mechanisms by which $p27^{Kip1}$ and $p21^{Cip1}$ block cell cycle progression remain to be defined, our findings suggest either that Cdk2 does not mediate the activity of these inhibitors or, alternatively, that cells possess compensatory mechanisms that efficiently bypass their requirement for Cdk2.

is haploinsufficient for tumor suppression (Fero et al., 1998). Cultured cells derived from *p27$^{Kip1-/-}$* mice show partial mitogen independence (Nakayama et al., 1996; Kiyokawa et al., 1996; Fero et al., 1996). In human tumors, the levels of p21^{Cip1} expression are not frequently altered. Yet, p21^{Cip1} expression cannot be induced in those tumors that do not express an active p53 tumor suppressor. The locus encoding p27^{Kip1} is also not mutated in most human tumors. However, the levels of p27^{Kip1} protein are frequently compromised in a variety of tumor types. This phenotype often correlates with increased tumor aggressiveness and poor prognosis (reviewed in Bloom and Pagano, 2003; Blain et al., 2003).

The observation that Cip/Kip inhibitors stabilize Cdk4/6-cyclin D complexes (Blain et al., 1997; LaBaer et al., 1997; Cheng et al., 1999) has led to the proposal that Cdk4/6-cyclin D kinases may contribute to cell cycle progression by sequestering Cip/Kip inhibitors away from other Cdks, mainly Cdk2. Decreased levels of p27^{Kip1} would generate sufficient amounts of active Cdk2-cyclin E complexes to phosphorylate p27^{Kip1}, a signal required for its degradation by the SCF-Skp2 proteasome (reviewed in Reed, 2003). Once most of p27^{Kip1} has been removed, fully active Cdk2-cyclin E complexes would be available to completely phosphorylate the Rb protein family, thus allowing cells to progress from the G1 to the S phase of the cell cycle (Sherr and Roberts, 1999; Reed 2003).

However, the central role attributed to Cdk2 in cell cycle progression has been recently challenged by the observation that mice lacking this kinase develop normally (Ortega et al., 2003; Berthet et al., 2003). Moreover, Cdk2-deficient cells proliferate well in culture and re-enter cell cycle after serum starvation without significant delay (Ortega et al., 2003). Presumably, ablation of Cdk2 is compensated by other Cdks. However, Cdk2 is essential for the first meiotic division of both male and female germ cells, an activity that cannot be compensated by any other kinase (Ortega et al., 2003). These findings have raised questions regarding other proposed roles for Cdk2 based on biochemical or cell biology studies. One such proposed role involves mediation of the cell cycle inhibitory and tumor suppressor activities of p27^{Kip1} and p21^{Cip1}.

In this study, we report that overexpression of p27^{Kip1} and p21^{Cip1} induces cell cycle arrest in the absence of Cdk2. Moreover, none of the deficiencies analyzed in *p27$^{Kip1-/-}$* and *p21$^{Cip1-/-}$* cells is reversed by deletion of Cdk2. Perhaps more importantly, ablation of Cdk2 does not reverse organomegalia *in p27$^{Kip1-/-}$* mice, nor prevent development of pituitary tumors. These results provide genetic evidence that Cdk2 is not an essential target for p21^{Cip1} or p27^{Kip1}. Moreover, they raise a note of caution regarding the suitability of Cdk2 as a target for therapeutic intervention, at least in those tumors lacking Cip/Kip tumor suppressors.

Results

p27^{Kip1} and p21^{Cip1} block cell cycle progression in the absence of Cdk2

To investigate whether Cip/Kip inhibitors cause cell cycle arrest in the absence of Cdk2, early-passage *Cdk2$^{+/+}$* and *Cdk2$^{-/-}$* mouse embryonic fibroblasts (MEFs) were infected with retroviruses expressing p27^{Kip1} or p21^{Cip1} (Figure 1A). Ectopic expression of p27^{Kip1} or p21^{Cip1} halted the proliferation of these primary MEFs regardless of the presence or absence of Cdk2 (Figure 1B). As expected, p27^{Kip1} induced cell cycle arrest in both G1 and G2/M, presumably by inhibiting the kinase activity of Cdk2 bound to cyclin E and cyclin A, respectively (Figure 1C). Surprisingly, p27^{Kip1} also blocked cell cycle progression in G1 (58% of the cells) and G2/M (42%) in the absence of Cdk2. It could be hypothesized that cell cycle arrest in G2/M may be mediated by Cdk1, a known target of p27^{Kip1} (reviewed in Pagano, 2004). However, the mechanism by which p27^{Kip1} induces cell cycle arrest in G1 in *Cdk2$^{-/-}$* cells is less obvious.

To investigate the effect of expressing p27^{Kip1} or p21^{Cip1} on various Cdk-cyclin complexes in the absence of Cdk2, we assayed their kinase activity present in the corresponding immunoprecipitates derived from *Cdk2$^{+/+}$* and *Cdk2$^{-/-}$* MEFs (Figure 2). As expected, whereas Cdk2 kinase activity was dramatically decreased in wild-type cells, ectopic expression of p27^{Kip1} or p21^{Cip1} had no effect on Cdk4 kinase, at least as determined

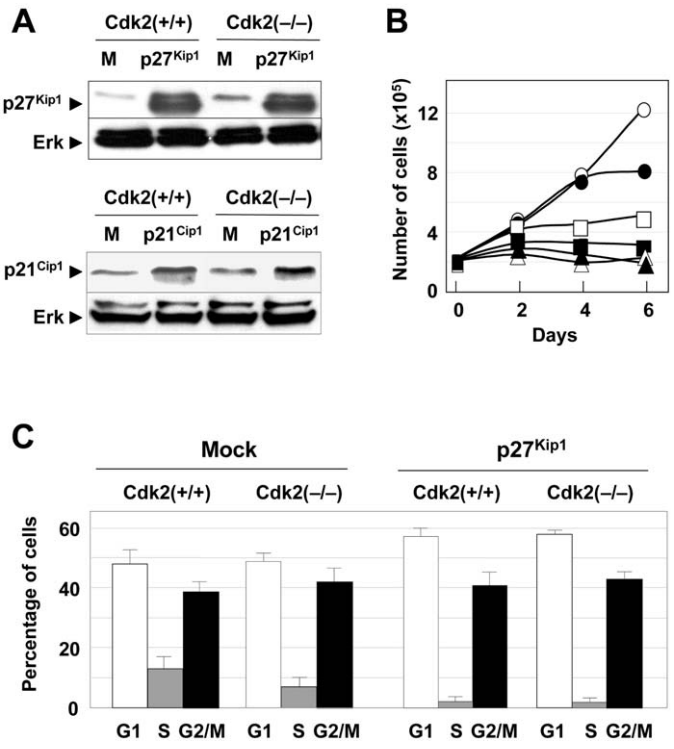

Figure 1. Ectopic expression of p27^{Kip1} and p21^{Cip1} arrests cell cycle progression in wild-type and Cdk2-deficient cells

A: Expression levels of p27^{Kip1} and p21^{Cip1} in *Cdk2$^{+/+}$* and *Cdk2$^{-/-}$* primary MEFs after retroviral infection with either empty virus (M) or with viruses encoding p27^{Kip1} or p21^{Cip1} proteins. Expression of the Erk protein was used as loading control. Migration of the proteins is indicated by arrowheads.

B: Growth curves of *Cdk2$^{+/+}$* (open symbols) and *Cdk2$^{-/-}$* (filled symbols) primary MEFs after retroviral infection with either empty virus (circles) or with viruses encoding p27^{Kip1} (triangles) or p21^{Cip1} (squares).

C: DNA content of *Cdk2$^{+/+}$* or *Cdk2$^{-/-}$* MEFs after infection with either empty virus (mock) or with a retrovirus encoding p27^{Kip1}. The percentage of cells in G1 (open bars), S (gray bars), or G2/M (solid bars) phases of the cell cycle is indicated. The means ± SD for 6 different embryos per genotype and 3 different experiments are shown.

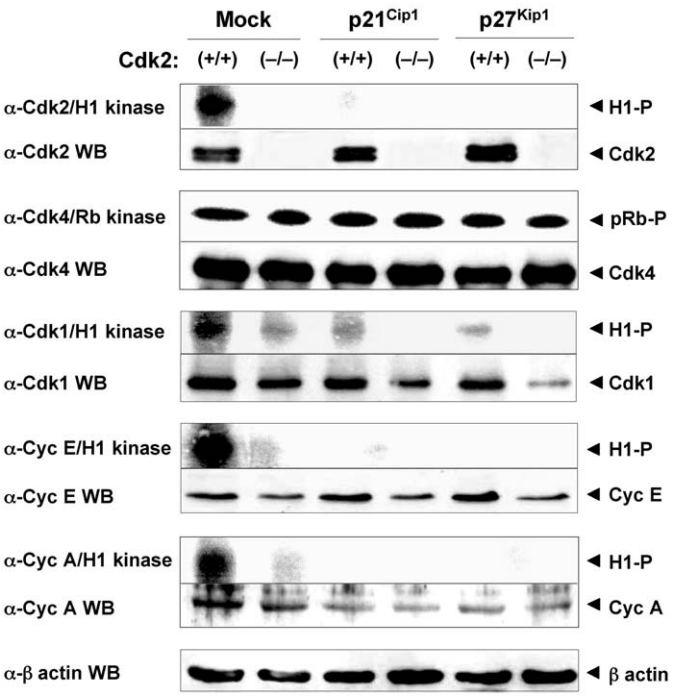

Figure 2. Expression levels and protein kinase activity of Cdk2, Cdk4, Cdk1, cyclin E, and cyclin A immunoprecipitates obtained from *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs ectopically expressing p27^{Kip1} or p21^{Cip1} proteins

Cell extracts obtained from *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs either mock infected or infected with retroviruses expressing p21^{Cip1} or p27^{Kip1} proteins were incubated with antibodies against Cdk2, Cdk4, Cdk1, cyclin E, and cyclin A, and the resulting immunoprecipitates assayed for kinase activity using histone H1 (H1 kinase) or a fragment of the retinoblastoma protein (Rb kinase) as substrates. Cell extracts were analyzed by Western blot with the same antibodies to determine the levels of expression of each of the above Cdks and cyclins. Results are depicted immediately below the corresponding kinase assay. The migration of the Cdks, cyclins, and phosphorylated substrates H1-P and pRb-P is indicated by arrowheads. A Western blot using β-actin antibodies was used as a loading control.

by its ability to phosphorylate pRb (Figure 2 and Supplemental Data). In agreement with previous studies, ectopic expression of p27^{Kip1} or p21^{Cip1} significantly reduced Cdk1 kinase activity in wild-type MEFs (Toyoshima and Hunter, 1994; Harper et al., 1995). In *Cdk2*$^{-/-}$ MEFs, Cdk1 kinase activity was completely inhibited upon p27^{Kip1} or p21^{Cip1} expression, a result that may account for the fraction of *Cdk2*$^{-/-}$ cells arrested in G2/M (Figure 1C) (Pagano, 2004). It should be noted that Cdk1 kinase activity was reduced in cells lacking Cdk2, most likely a consequence of the lower levels of Cdk1 expression in Cdk2 null cells (Ortega et al., 2003). Cyclin E-associated kinase activity, presumably mediated by Cdk2, was also inhibited by p27^{Kip1} or p21^{Cip1} expression regardless of whether we used histone H1 or pRB protein as a substrate (see Figure 2 and Supplemental Data). No cyclin E associated kinase activity could be observed in immunoprecipitates obtained from *Cdk2*$^{-/-}$ cells, regardless of the expression of p27^{Kip1} or p21^{Cip1} (Ortega et al., 2003). In agreement with previous studies (Ortega et al., 2003; Berthet et al., 2003), Cyclin A-associated kinase activity is greatly decreased in *Cdk2* null cells (Figure 2). This residual activity, presumably mediated by Cdk1-cyclin A complexes, was completely eliminated upon expression of p27^{Kip1} or p21^{Cip1} (Figure 2), thus providing further support to the concept that these inhibitors also block Cdk1 kinase activity.

Biological activity of mutant p27^{Kip1} and p21^{Cip1} proteins is independent of Cdk2

To determine whether binding of p27^{Kip1} to Cdk-cyclin complexes is required for its cell cycle inhibitory properties in *Cdk2* null cells, we generated two p27^{Kip1} mutants, p27^{Kip1} F64A and p27^{Kip1}3M. p27^{Kip1}F64A has a single F64A mutation that reduces binding to the Cdk catalytic subunit (Kwon and Nordin, 1998). p27^{Kip1}3M carries two additional mutations (L32H;P35A) within a putative cyclin binding motif based on sequence homology with p21^{Cip1} (Lin et al., 1996). Thus, p27^{Kip1}3M should not be able to bind either Cdk or cyclin subunits. As illustrated in Figure 3A, the p27^{Kip1}F64A mutant binds to all Cdk and cyclin subunits examined with an efficiency similar to that of the wild-type protein. Whether recognition of the catalytic subunits Cdk1, Cdk2, and Cdk4 was mediated by binding to their cognate cyclins was not examined. In contrast, the triple mutant, p27^{Kip1}3M, was not able to recognize any Cdk-cyclin complexes as determined by immunoprecipitation assays (Figure 3B). Next, we examined whether these mutant p27^{Kip1} proteins retained cell cycle inhibitory properties. As illustrated in Figure 3C, p27^{Kip1}F64A was almost as efficient as the wild-type protein in blocking cell cycle progression as determined by BrdU incorporation. Its inhibitory activity was the same in *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs. In contrast, p27^{Kip1}3M completely lost its ability to inhibit BrdU incorporation in both wild-type and *Cdk2*$^{-/-}$ MEFs. These observations indicate a strong correlation between cell cycle inhibitory activity of p27^{Kip1} and its ability to interact with Cdk-cyclin complexes, regardless of the presence or absence of Cdk2.

In addition to Cdk-cyclin complexes, p21^{Cip1} also binds to PCNA, preventing its interaction with the catalytic subunit of DNA polymerase delta, an enzyme required for DNA replication (Luo et al., 1995; Chen et al., 1995). Expression of p21$^{Cip1}\Delta$, a mutant p21^{Cip1} protein that cannot bind to PCNA (Cayrol et al., 1998), also caused cell cycle arrest in MEFs regardless of Cdk2 expression (see Supplemental Data). These results suggest that PCNA is not a major target for p21^{Cip1}, at least in MEFs.

Loss of Cdk2 does not suppress the proliferative advantages of cells lacking p27^{Kip1} and p21^{Cip1}

To provide genetic evidence for a possible epistatic interaction between Cip/Kip inhibitors and Cdk2, we examined the consequences of ablating Cdk2 in cells defective for either p27^{Kip1} or p21^{Cip1}. Double knockout *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ and *p21*$^{Cip1-/-}$*;Cdk2*$^{-/-}$ mice were born at the expected Mendelian ratio and did not display any defects other than those previously reported for their parental single knockout animals. Primary *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ and *p21*$^{Cip1-/-}$*;Cdk2*$^{-/-}$ MEFs, isolated from mid-gestation embryos, displayed increased proliferation rates similar to those of *p27*^{Kip1} null or *p21*^{Cip1} null cells (Figure 4A), thus indicating that loss of Cdk2 does not abrogate the proliferative advantage conferred by the absence of p27^{Kip1} or p21^{Cip1}. Similar results were obtained in colony formation assays. For instance, whereas we scored 25 ± 14 colonies after seeding 3,000 wild-type cells, *p21*$^{Cip1-/-}$ MEFs yielded 114 ± 16 colonies, a figure very similar to that obtained in the corresponding MEFs lacking both p21^{Cip1} and Cdk2 (118 ± 13 colo-

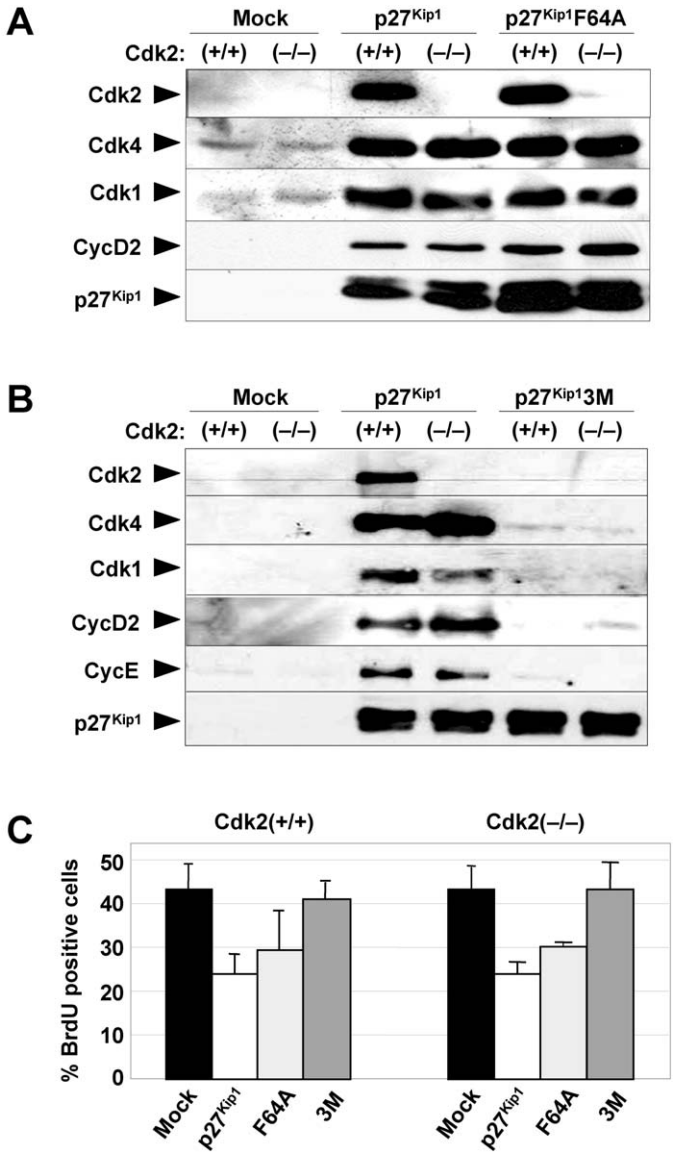

Figure 3. Cell cycle inhibitory properties of mutant p27^{Kip1} proteins

A: *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs infected with empty retrovirus (mock) or with viruses expressing Flag-tagged wild-type p27^{Kip1} or mutated p27^{Kip1}F64A proteins.

B: *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs infected with empty retrovirus (mock) or with viruses expressing Flag-tagged wild-type p27^{Kip1} or mutated p27^{Kip1}3M proteins. p27^{Kip1} complexes were immunoprecipitated using anti-Flag antibodies, and the levels of the indicated proteins, including Cdk2, Cdk4, Cdk1, cyclin D2, cyclin E, and p27^{Kip1}, were detected by Western blot analysis using the corresponding antibodies. Migration of these proteins is indicated by arrowheads.

C: BrdU incorporation (expressed as percentage of positive cells) in *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ immortal MEFs either mock infected (Mock; filled bars) or infected with retroviruses encoding Flag-tagged p27^{Kip1} wild-type (p27^{Kip1}; open bars) or mutated Fg-p27^{Kip1}F64A (F64A; light gray bars) and Fg-p27^{Kip1}3M (3M; dark gray bars) proteins. The average value of two different clones per genotype is represented. At least two different experiments were performed with all the cell lines.

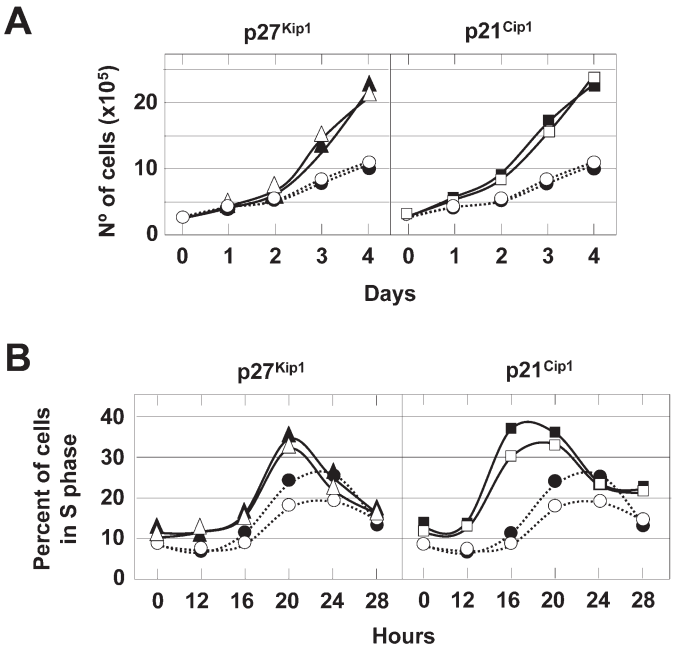

Figure 4. Ablation of Cdk2 does not affect the proliferative properties of cells lacking p27^{Kip1} or p21^{Cip1} inhibitors

A: Proliferation rates of primary MEFs deficient in p27^{Kip1} (triangles) or p21^{Cip1} (squares) in the presence (closed symbols) or absence (open symbols) of Cdk2. Curves depicting the proliferation rates of wild-type (closed circles) and *Cdk2*$^{-/-}$ (open circles) primary MEFs are also indicated by dotted lines.

B: Kinetic analysis of S phase entry of primary MEFs deficient in p27^{Kip1} or p21^{Cip1} in the presence or absence of Cdk2. Kinetics of S phase entry of wild-type and Cdk2 primary MEFs are also indicated. Symbols are those indicated in **A**.

nies). Finally, quiescent p27^{Kip1} and p21^{Cip1} deficient MEFs enter S phase 4 to 6 hr earlier than wild-type cells upon serum stimulation, a differential effect also observed in primary *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ and *p21*$^{Cip1-/-}$*;Cdk2*$^{-/-}$ MEFs (Figure 4B), suggesting that the shortening in S phase entry is not mediated by constitutively active Cdk2.

Cdk2 is not essential for p21^{Cip1}-mediated cell cycle arrest after DNA damage

p21^{Cip1} is one of the major effectors of cell cycle arrest induced upon DNA damage (Deng et al., 1995; Brugarolas et al., 1995). To examine the contribution of Cdk2 to these DNA damage checkpoints, we exposed wild-type, *Cdk2*$^{-/-}$, *p21*$^{Cip1-/-}$, and *p21*$^{Cip1-/-}$*;Cdk2*$^{-/-}$ primary MEFs to γ irradiation or to etoposide. Serum-starved cells were irradiated in suspension using a single dose of 10 Gy and stimulated with 10% of serum to reenter the cell cycle. BrdU was added to allow detection of cells entering S phase. Irradiated wild-type and *Cdk2*$^{-/-}$ MEFs showed a 60% reduction in the number of BrdU-positive cells and a concomitant 10%–15% increase in the G1 population relative to nonirradiated samples (Figure 5A). Ablation of p21^{Cip1} resulted in impaired checkpoint arrest, since *p21*^{Cip1} null cells maintained high levels of BrdU incorporation and lacked G1 arrest. As illustrated in Figure 5A, double mutant *p21*$^{Cip1-/-}$*;Cdk2*$^{-/-}$ cells also displayed increased BrdU incor-

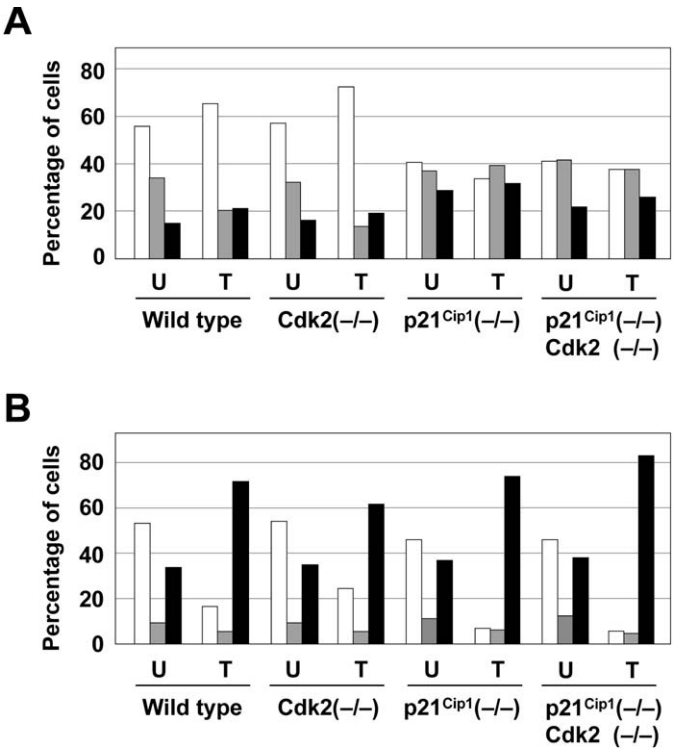

Figure 5. DNA damage checkpoint in MEFs exposed to γ radiation and etoposide

A: Percentage of cells either untreated (U) or exposed (T) to γ radiation in G1 (open bars), S (gray bars), or G2/M (filled bars) phases of the cell cycle. The corresponding genotype of each of the treated cells is indicated.
B: As above except that cells were treated with etoposide.

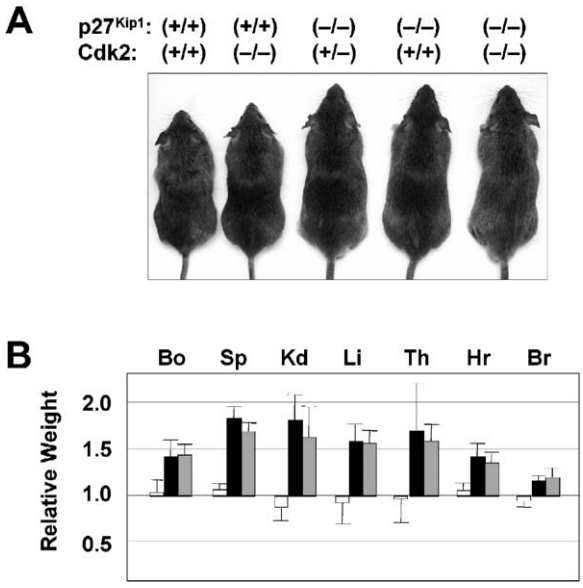

Figure 6. Loss of Cdk2 does not abrogate organomegalia of *p27*^{Kip1} null mice

A: Mice lacking p27^{Kip1} have larger size regardless of the presence or absence of Cdk2. The genotypes of the representative mice depicted in this photograph are indicated.
B: Total body weight (Bo) and weight of individual organs, including spleen (Sp), kidney (Kd), liver (Li), thymus (Th), heart (Hr), and brain (Br) of *Cdk2*$^{-/-}$ (open bars), *p27*$^{Kip1-/-}$ (black bars), and *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ (gray bars) mice relative to the corresponding weights of wild-type mice. The means ± SD for 3 different animals from each genotype are shown.

poration and failed to arrest in G1. Similar results were obtained using etoposide, another DNA damaging agent (Figure 5B). Whereas wild-type cells arrested both in G1 and G2/M, *p21*^{Cip1} null cells treated with etoposide were not able to arrest in G1, and most of them accumulated in G2/M. As illustrated in Figure 5B, this effect was also independent of Cdk2. These observations provide genetic evidence against the concept that p21^{Cip1} mediates cell cycle arrest at the DNA damage checkpoint by blocking Cdk2 activity.

Cip/Kip tumor suppressor activity does not require Cdk2

p27^{Kip1} and p21^{Cip1}-deficient MEFs show slightly increased susceptibility to cellular transformation in vitro. Ectopic expression of Ras and E1A oncogenes induced significantly more foci of transformed cells in either *p27*^{Kip1} and *p21*^{Cip1} null MEFs than in wild-type cells. Whereas we scored 17 ± 8 foci in wild-type cells, we observed 20 ± 7 and 33 ± 7 in p27^{Kip1} and p21^{Cip1} null MEFs, respectively. When similar experiments were carried out using primary MEFs lacking Cdk2, results were essentially identical. That is, we observed 23 ± 9 foci in *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ cells, and 27 ± 5 foci in *p21*$^{Cip1-/-}$*;Cdk2*$^{-/-}$ MEFs, respectively. These observations suggest that the increased susceptibility to transformation by Ras and E1A oncogenes of Cip/Kip deficient cells is not due to increased Cdk2 activity.

To examine the genetic interactions between Cdk2 and Cip/Kip inhibitors in vivo, we analyzed the phenotype of mice deficient for Cdk2 and p27^{Kip1}. Ablation of *p27*^{Kip1} in mice results in hyperplasia leading to generalized organomegaly and increased body size. Moreover, these animals develop retinal dysplasia and pituitary tumors of the intermediate lobe (Nakayama et al., 1996; Kiyokawa et al., 1996; Fero et al., 1996). *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ double mutant mice weighted 50% to 60% more than wild-type and *Cdk2*$^{-/-}$ mice and displayed widespread organomegalia, similar to *p27*$^{Kip1-/-}$ animals (Figure 6). Testes and ovaries of *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ mutant mice also displayed reduced size similar to those of *Cdk2* null mice (data not shown). Close examination of these tissues revealed complete absence of germ cells as well as an atrophic architecture indistinguishable from that of *Cdk2*$^{-/-}$ single mutant mice (Ortega et al., 2003) (data not shown). These observations indicate that the meiotic defects that result from ablation of Cdk2 are independent of p27^{Kip1}.

p27$^{Kip1-/-}$*;Cdk2*$^{-/-}$ mice displayed the same retinal defects observed in *p27*^{Kip1} null mice and with similar low penetrance (about 10%) (Nakayama et al., 1996; Kiyokawa et al., 1996). These defects involve partial invasion of the rods and cones layer by the outer granular layer (Figure 7A). More importantly, these double mutant mice develop pituitary tumors with the same penetrance and latency as p27^{Kip1} single mutant animals. As illustrated in Figure 7B, 10-week-old *p27*$^{Kip1-/-}$ (n = 3) and *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$mice (n = 3) displayed similar hyperplastic pituitary glands. Moreover, all *p27*$^{Kip1-/-}$ (n = 6) and *p27*$^{Kip1-/-}$*;Cdk2*$^{-/-}$ (n = 6), but not *Cdk2*$^{-/-}$ (n = 6), mice had

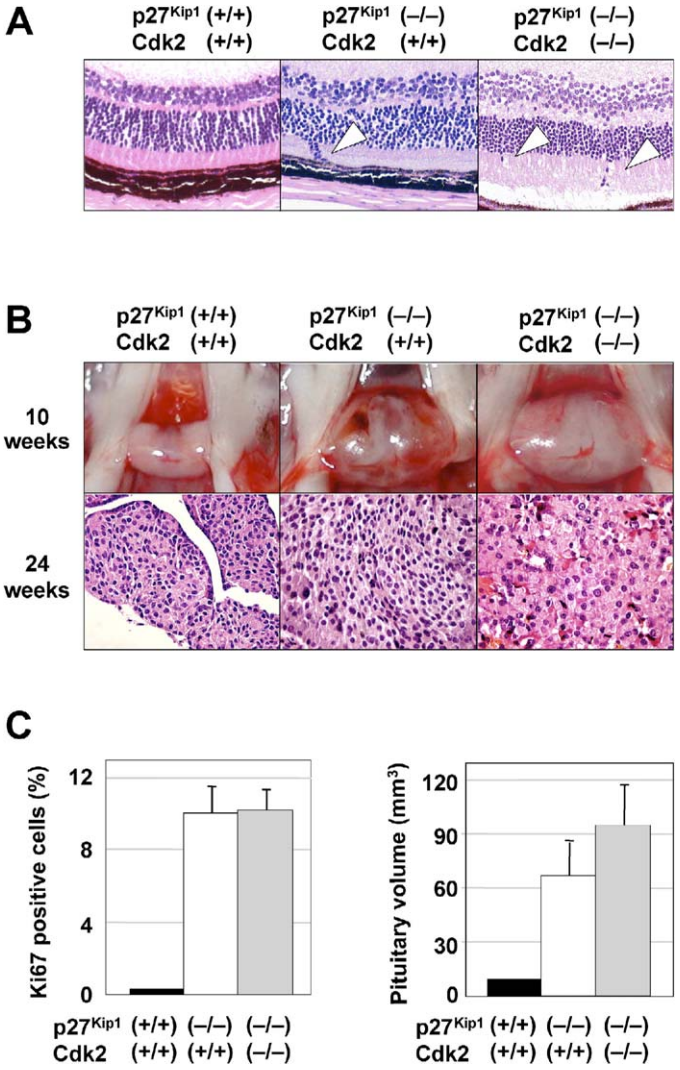

Figure 7. Loss of Cdk2 does not suppress the tumor suppressor properties of p27^{Kip1}

A: H&E staining of sections of normal retinas obtained from *p27$^{Kip1+/+}$;Cdk2$^{+/+}$* mice and of retinas obtained from *p27$^{Kip1-/-}$;Cdk2$^{+/+}$* and *p27$^{Kip1-/-}$;Cdk2$^{-/-}$* mice displaying the invasion of the rod and cone layer (arrowheads) by the outer granular layer.

B: Upper row: 10-week-old mice. Photographs of normal pituitary glands obtained from *p27$^{Kip1+/+}$;Cdk2$^{+/+}$* mice and hyperplastic glands observed in *p27$^{Kip1-/-}$;Cdk2$^{+/+}$* and *p27$^{Kip1-/-}$;Cdk2$^{-/-}$* animals. Lower row: 24-week-old mice. H&E staining of sections of normal pituitary glands obtained from *p27$^{Kip1+/+}$;Cdk2$^{+/+}$* mice and from pituitary tumors that developed in *p27$^{Kip1-/-}$;Cdk2$^{+/+}$* and *p27$^{Kip1-/-}$;Cdk2$^{-/-}$* double mutant mice.

C: Percentage of Ki67-positive cells and size of pituitary glands from 24-week-old *p27$^{Kip1+/+}$;Cdk2$^{+/+}$* (filled bars), *p27$^{Kip1-/-}$;Cdk2$^{+/+}$* (open bars), and *p27$^{Kip1-/-}$;Cdk2$^{-/-}$* (gray bars) mice. All pituitary glands examined from *p27$^{Kip1-/-}$;Cdk2$^{+/+}$* and *p27$^{Kip1-/-}$;Cdk2$^{-/-}$* mice were neoplastic. Pituitary sections derived from five 6 month-old mice were analyzed. At least 3 different fields per section were counted and the resulting means ± SD are represented.

pituitary tumors at six months of age (Figure 7B). These pituitary tumors display similar elevated proliferative indexes and volumes in *p27$^{Kip1-/-}$* and *p27$^{Kip1-/-}$;Cdk2$^{-/-}$* double mutant mice (Figure 7C). These results illustrate that Cdk2 does not play a significant role in mediating the in vivo tumor suppressor activity of p27^{Kip1}.

Discussion

We and others have recently demonstrated that Cdk2 is not essential for the mitotic cell cycle in mammalian cells (Ortega et al., 2003; Berthet et al., 2003). Now, we describe results that establish that Cdk2 is not essential for mediating either the cell cycle inhibitory or the tumor suppressing properties of p27^{Kip1} and p21^{Cip1}. Moreover, our results argue against an epistatic relationship between these Cip/Kip inhibitors and Cdk2, thus challenging one of the most widely established steps in the progression from the G1 to the S phase of the cell cycle.

Our findings illustrate that p27^{Kip1}and p21^{Cip1} can inhibit cell cycle progression in cells that lack Cdk2. These observations suggest either that Cdk2 is not the primary target of Cip/Kip inhibitors in vivo, or, alternatively, that p27^{Kip1}and p21^{Cip1} can block cell cycle progression by interacting with molecules other than Cdk2. Assuming the latter, the most likely compensatory molecules would be the Cdks involved in cell cycle progression, mainly Cdk1, Cdk3, Cdk4, and Cdk6. In fact, p27^{Kip1} mutant proteins defective in Cdk and cyclin binding are not effective in blocking cell cycle in *Cdk2* null cells.

Cdk4 and Cdk6 are unlikely mediators of the cell cycle inhibitory properties of p27^{Kip1}and p21^{Cip1}, since it has been well illustrated that these inhibitors do not block the kinase activity of Cdk4/6-cyclin D complexes. Instead, increasing evidence supports the concept that Cip/Kip inhibitors help to stabilize Cdk4/6-cyclin D complexes (Blain et al., 1997; LaBaer et al., 1997; Cheng et al., 1999). Recent genetic evidence also argues against a compensatory role of Cdk4 and Cdk6 in mediating p27^{Kip1}and p21^{Cip1} activities, since both of these molecules effectively block cell proliferation in MEFs lacking either Cdk4 and Cdk6, or the three D type cyclins (Malumbres et al., 2004; Kozar et al., 2004). Thus, it is unlikely that Cdk4 and Cdk6 mediate cell cycle inhibition by Cip/Kip inhibitors. On the other hand, we have recently shown, using shRNA strategies, that Cdk2 partially compensates for the absence of Cdk4 and Cdk6 in MEFs, most likely by a mechanism involving its interaction with D type cyclins (Malumbres et al., 2004). Thus, it is possible that if in cells lacking Cdk4 and Cdk6, Cdk2-cyclin D complexes are responsible for driving cells through G1, p27^{Kip1} and p21^{Cip1} may block cell cycle progression by inhibiting Cdk2-cyclin D kinase activity. Yet, this hypothesis would not explain how p27^{Kip1} and p21^{Cip1} inhibit the cell cycle in the absence of Cdk2.

Cdk3, a Cdk2-related kinase recently implicated in the G0/G1 transition (Ren and Rollins, 2004), is unlikely to serve as a compensatory molecule for Cdk2, since it is not functional in all the cells and mice used in this study (data not shown) due to a naturally occurring mutation in the *cdk3* locus of most laboratory strains of mice (Ye et al., 2001).

Cdk1 is also a candidate to compensate for the absence of Cdk2, since p27^{Kip1} and p21^{Cip1} bind to Cdk1 and inhibit its kinase activity (Toyoshima and Hunter, 1994; Harper et al., 1995), thus making Cdk1 a potential candidate to compensate for the absence of Cdk2. In agreement with these observations, we have detected p27^{Kip1} and p21^{Cip1} binding to Cdk1 and inhibition of its kinase activity in both wild-type and Cdk2-deficient cells (Figure 2). Moreover, a significant percentage of

Cdk2-deficient cells arrest in the G2/M phase of the cell cycle upon ectopic expression of $p27^{Kip1}$, thus suggesting that Cdk1 mediates at least some of the properties of these inhibitors in the absence of Cdk2. This hypothesis is supported by recent results demonstrating that ablation of $p27^{Kip1}$ suppresses endoreplication of Skp2-deficient hepatocytes, an observation attributed to inhibition of Cdk1 by increased expression of $p27^{Kip1}$ during the S/G2 transition (Nakayama et al., 2004). However, these observations do not explain how $p27^{Kip1}$ and $p21^{Cip1}$ effectively block cell cycle progression in G1 in the absence of Cdk2. Indeed, if we postulate that Cdk1 mediates all the cell cycle inhibitory activity of $p27^{Kip1}$ and $p21^{Cip1}$ in the absence of Cdk2, such a hypothesis would imply that Cdk1 must participate in driving the G1/S transition, a possibility that deserves further examination. To date, we have not been able to obtain Cdk1-deficient MEFs, since mice lacking this kinase die during the very early stages of embryonic development (our unpublished observations). Whether generation of Cdk1 conditional mutant cells or even shRNA approaches would help to resolve this issue remains to be determined.

Regardless of the molecular mechanisms responsible for the activity of $p27^{Kip1}$ in cells lacking Cdk2, we find it remarkable that ablation of Cdk2 did not revert any of the phenotypes induced by loss of this tumor suppressor. These observations suggest either that Cdk2 may not be a physiological target of $p27^{Kip1}$ or that mice may have additional targets equally responsible for mediating the tumor suppressor properties of $p27^{Kip1}$ (Pagano, 2004). These findings are in sharp contrast with those recently reported for mice deficient for $p27^{Kip1}$ and Skp2 (Nakayama et al., 2004). Concomitant ablation of these molecules restores most of the defects observed in *$p27^{Kip1}$* null animals, thus establishing an epistatic relationship between $p27^{Kip1}$ and Skp2, the F box protein responsible for its degradation (Nakayama et al., 2004).

Loss of $p27^{Kip1}$ expression is a common feature of many human cancers, an event frequently associated with poor prognosis (reviewed in Bloom and Pagano, 2003; Blain et al., 2003; Malumbres and Carnero, 2003). Likewise, a large fraction of human neoplasias carry mutations in the p53 pathway that prevent expression of $p21^{Cip1}$ (reviewed in Lowe et al., 2004; Vousden and Prives, 2005). Although we realize that Cdk2 inhibition by selective inhibitors might have different effects than eliminating the Cdk2 protein, our findings raise a note of caution regarding the suitability of Cdk2 as a target for therapeutic intervention, at least in tumors lacking $p27^{Kip1}$ or $p21^{Cip1}$ expression. In any case, the results reported here should stimulate further work to identify essential mediator(s) of the tumor suppressor properties of the $p27^{Kip1}$ and $p21^{Cip1}$ cell cycle inhibitors.

Experimental procedures

Mice and tissues

$p27^{Kip1-/-}$, *$p21^{Cip1-/-}$*, and *$Cdk2^{-/-}$* mice have been described (Brugarolas et al., 1995; Kiyokawa et al., 1996; Ortega et al., 2003). Tissue samples were fixed in formalin for 24 hr and embedded in paraffin, and 3 μm sections were analyzed after staining with hematoxylin and eosin (H&E). For proliferation studies, tissues were stained with Ki67-specific antibodies (Dako). All experiments involving mice were performed in accordance with institutional guidelines (CNIO Committee for the Use and Care of Experimental Animals) and the corresponding national regulations.

Cell culture

Primary MEFs were obtained from E13.5 embryos as reported (Ortega et al., 2003). For cell proliferation assays, we plated 5×10^4 cells on six-well plates and counted daily for 4 days. For S phase assays, P2 MEFs (10^6 cells per 10 cm dish) were placed in DMEM containing 0.1% FBS for 60 hr. Cells were restimulated by addition of 10% FBS. 50 μM of BrdU was added 1 hr before harvesting at the indicated time points. DNA content was analyzed by flow cytometry (Coulter XL or FACScan from Becton-Dickinson). Focus formation assays were performed as described (Sotillo et al., 2001). For γ irradiation, MEFs were trypsinized and suspended in growth medium before irradiation at room temperature with a dose of 10 Gy. Cells were replated in growth medium at 40%–60% confluence. The percentage of cells in S phase was determined by flow cytometric analysis and by BrdU incorporation. For etoposide treatment, MEFs were grown in the presence of 5 μM etoposide for 24 hr and collected 1 day later. Cell cycle profile was also determined using flow cytometry. For expression of $p27^{Kip1}$ or $p21^{Cip1}$, phoenix cells were transfected with 6 μg of ecotropic helper retrovirus plasmid (pCL-Eco) plus 6 μg of pBabe vector encoding the corresponding proteins. The $p21^{Cip1}$ mutant cDNA defective for PCNA binding ($p21^{Cip1}\Delta$) was described previously (Cayrol et al., 1998). A Flag-tagged $p27^{Kip1}$ cDNA was modified using in vitro mutagenesis (Stratagene) to obtain two cDNAs encoding a single (F64A) and a triple (L32H;P35A;F64A) mutant designated as $p27^{Kip1}$F64A and $p27^{Kip1}$3M, respectively (Lin et al., 1996; Kwon and Nordin, 1998). Supernatants were used to infect primary MEFs as described (Cheng et al., 1999). Cells were placed under puromycin selection and harvested 2–3 days later.

Western blot, immunoprecipitation, and kinase assays

Protein isolation and analysis was performed as described (Ortega et al., 2003). Protein extracts were immunoprecipitated by using antibodies against Flag (Sigma), Cdk1, Cdk2, Cdk4, cyclin E, or cyclin A (Santa Cruz Biotechnology). For Western blot analysis, protein lysates were transferred to nitrocellulose membranes and probed with antibodies against Cdk2, Cdk1, Cdk4, $p21^{Cip1}$, cyclin E, cyclin A, cyclin D2, or ERK (Santa Cruz Biotechnology), $p27^{Kip1}$ (Transduction Laboratories), or β-actin (Sigma). For kinase assays, we used 1 μg of mouse pRb protein fragment (amino acids 769–921; Santa Cruz Biotechnology) or histone H1 (Roche) as substrates.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/6/591/DC1/.

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