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Mammalian Cells Cycle without the D-Type Cyclin-Dependent Kinases Cdk4 and Cdk6

Marcos Malumbres,[1,*] Rocío Sotillo,[1] David Santamaría,[1] Javier Galán,[1] Ana Cerezo,[1] Sagrario Ortega,[1,3] Pierre Dubus,[2] and Mariano Barbacid[1,*]
[1]Molecular Oncology Program
Centro Nacional de Investigaciones Oncológicas (CNIO)
E-28029 Madrid
Spain
[2]EA2406 University of Bordeaux 2
F-33076 Bordeaux
France

Summary

Cdk4 and Cdk6 are thought to be essential for initiation of the cell cycle in response to mitogenic stimuli. Previous studies have shown that Cdk4 is dispensable for proliferation in most cell types, an observation attributed to a putative compensatory role by Cdk6. *Cdk6*-null mice are viable and develop normally although hematopoiesis is slightly impaired. Embryos defective for Cdk4 and Cdk6 die during the late stages of embryonic development due to severe anemia. However, these embryos display normal organogenesis and most cell types proliferate normally. In vitro, embryonic fibroblasts lacking Cdk4 and Cdk6 proliferate and become immortal upon serial passage. Moreover, quiescent Cdk4/Cdk6-null cells respond to serum stimulation and enter S phase with normal kinetics although with lower efficiency. These results indicate that D-type cyclin-dependent kinases are not essential for cell cycle entry and suggest the existence of alternative mechanisms to initiate cell proliferation upon mitogenic stimulation.

Introduction

Progression throughout the mammalian cell cycle is driven by heterodimeric kinases made of a regulatory subunit, designated as cyclin, and a catalytic subunit known as cyclin-dependent kinase or Cdk. Two of these Cdks, Cdk4 and Cdk6, bind to and are activated by the D-type family of cyclins, D1, D2, and D3 (reviewed in Sherr and Roberts [1999]). In their active form, these kinases phosphorylate members of the retinoblastoma (Rb) protein family, pRb, p107, and p130. The Rb family of proteins are tumor suppressors that in their active, nonphosphorylated form prevent expression of genes necessary for DNA replication (S phase of the cell cycle) and mitosis (M phase) (reviewed in Classon and Harlow [2002]). In quiescent cells, Rb proteins repress transcription by a complex mechanism that involves inactivation of transcription factors such as the E2F family and binding to histone deacetylases and chromatin remodeling proteins (reviewed in Harbour and Dean [2000]; Stevaux and Dyson [2002]). The complexity of Rb function is highlighted by the fact that pRb can bind more than 120 cellular proteins (Morris and Dyson, 2001).

Phosphorylation of pRb by D-type cyclin/Cdk4 or Cdk6 complexes results in its partial inactivation, which in turn allows expression of a limited number of transcriptional targets needed to drive cells through the G1 phase of the cell cycle. For instance, some of these targets are additional cyclins, such as the E-type cyclins (E1 and E2), whose primary role is to activate Cdk2. Active E-type cyclin/Cdk2 complexes further phosphorylate pRb (Lundberg and Weinberg, 1998; Harbour et al., 1999) leading to a wave of transcriptional activity essential to proceed through the G1/S transition. However, recent genetic evidence indicating that Cdk2 and E-type cyclins are not required for mitotic cell division has challenged this model (Berthet et al., 2003; Ortega et al., 2003; Geng et al., 2003; Parisi et al., 2003). Additional work will be necessary to determine the precise role of Cdk2 and E-cyclins as well as the contribution of other compensatory kinases before the molecular events that regulate the G1/S transition are fully understood.

The molecular link between mitogenic signaling and cell cycle progression started to take shape when several groups reported the induction of D-type cyclins after growth factor signaling or Ras oncogenic activity (reviewed in Stacey and Kazlauskas [2002]). Moreover, the *cyclin D1* promoter contains sequences recognized by several transcription factors such as AP-1 or NF-κB, known to be activated upon mitogenic signaling (reviewed in Amanatullah et al. [2001]). These and other related observations have led to the widely held hypothesis that D-type cyclins, in complex with Cdk4 or Cdk6, are the molecular sensors that activate cell cycle progression in response to mitogenic stimuli (Sherr and Roberts, 1999). Likewise, antimitogenic signals, such as contact inhibition, senescence, or exposure to TGF-β, frequently inactivate the formation of D-type cyclin/Cdk4 or Cdk6 complexes by inducing expression of a family of cell cycle inhibitors known as INK4 (*In*hibitor of Cd*k4*). The members of this protein family, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, are competitive inhibitors of D-type cyclins for binding to Cdk4 and Cdk6 (reviewed in Pavletich [1999]; Sherr and Roberts [1999]).

The relevance of D-type cyclin/Cdk4 and Cdk6 complexes in the control of cell proliferation has been underscored by their frequent deregulation in human cancer. Most, if not all human tumor cells have altered their D-type cyclin-Cdk4/6-INK4-pRb pathway either by mutations that activate D-type cyclin/Cdk4 or Cdk6 activity or by mutations that eliminate INK4 or pRb (reviewed in Malumbres and Barbacid [2001]). These observations suggest that mutations that render cells less dependent of growth factor requirements for cell cycle progression are more likely to become tumorigenic. Likewise, these mutations presumably make cells less responsive to antimitogenic signals, one of the hallmarks of cancer cells (reviewed in Hanahan and Weinberg [2000]).

*Correspondence: malumbres@cnio.es (M.M.), barbacid@cnio.es (M.B.)
[3]Present address: Biotechnology Program, CNIO, E-28029 Madrid, Spain.

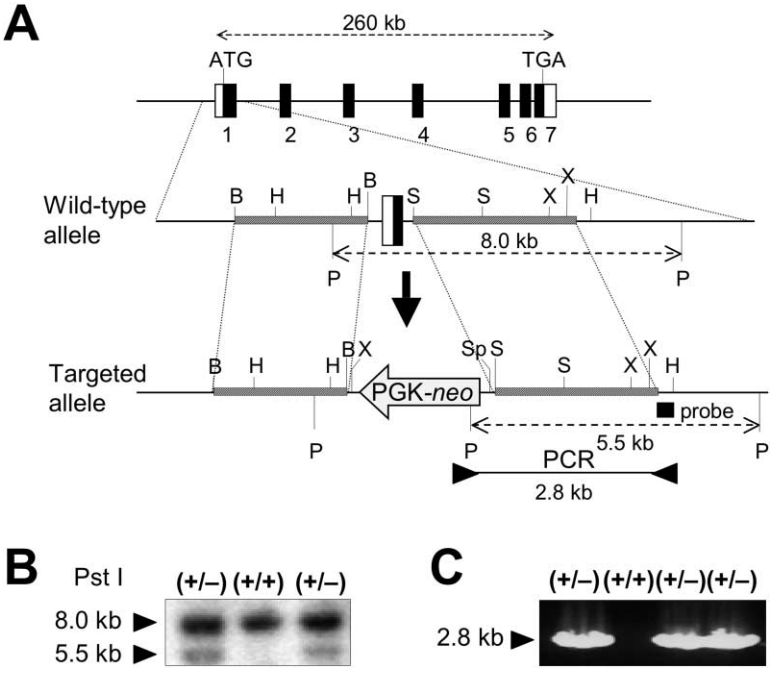

Figure 1. Gene Targeting of the Mouse *Cdk6* Locus

(A) Gene targeting strategy. The mouse *Cdk6* locus contains seven exons (boxes) spanning about 260 kbp. Noncoding sequences (open boxes), coding sequences (filled boxes), and translational initiator (ATG) and terminator (TGA) codons are indicated. Sequences present in the targeting vector are indicated by a thick hatched bar. The phosphoglycerol kinase promoter (PGK)-neomycin resistance gene (*neo*) cassette is indicated by a gray box. The arrow indicates the transcriptional orientation of the cassette. The probe used to identify the diagnostic PstI (P) fragments is indicated by a thick bar. Other restriction sites include BamHI (B), HindIII (H), SAII (S), XbaI (X), and SphI (Sp). The position of oligonucleotides used for PCR analysis of the recombinant allele are indicated by triangles. Representative (B) Southern blot and (C) PCR analysis of recombinant ES cell clones carrying the indicated alleles is shown. Migration of the corresponding DNA fragments is indicated by arrowheads.

However, these observations do not establish whether D-type cyclins and/or their cognate catalytic partners, Cdk4 and Cdk6, are the only elements that mediate the cell cycle response to mitogenic and antimitogenic signaling. In an effort to address this question, gene-targeted mice deficient for each, or even two, D-type cyclins have been generated (Fantl et al., 1995; Sicinski et al., 1995, 1996; Ciemerych et al., 2002; Sicinska et al., 2003). In general, D-type cyclins have overlapping roles in those tissues where more than one family member is expressed. Similarly, mice deficient in Cdk4 (Rane et al., 1999; Tsutsui et al., 1999) are viable and only display proliferative defects in specific endocrine cell types (Martín et al., 2003; Moons et al., 2002). Whereas at least some of these defects could be attributed to the absence of Cdk6 expression in the affected cell types, it is unlikely that the lack of widespread proliferative defects in Cdk4-null animals can be solely explained by the compensatory activity of Cdk6.

To analyze the physiological roles of Cdk4 and Cdk6 in vivo as well as to dissect their specific contributions to cell cycle progression after mitogenic stimuli, we have generated mice defective for Cdk6 as well as double mutant mice lacking both Cdk4 and Cdk6. Here, we report that Cdk6-deficient mice are viable and display mild defects in defined hematopoietic cell populations. Double deficiency in Cdk4 and Cdk6 results in either late embryonic or postnatal lethality, most likely due to a defect in the erythroid lineage that results in severe anemia. However, Cdk4- and Cdk6-null embryos display normal organogenesis and cell proliferation levels. We also report here that Cdk4 and Cdk6 double mutant mouse embryonic fibroblasts (MEFs) proliferate in response to serum stimulation and become immortalized upon continuous passage. These observations suggest that D-type cyclin-dependent kinases are not essential for cell cycle progression of quiescent cells upon exposure to mitogenic stimuli.

Results

Generation of Cdk6 Knockout Mice

To ablate the functional mouse *Cdk6* locus, we replaced its first coding exon with a PGK-*neo* cassette by homologous recombination in embryonic stem (ES) cells. This exon encodes the ATG initiation codon and the PLSTIRE domain essential for cyclin binding (Figure 1). Identification of recombinant *neo*^{R} ES cell clones was determined by DNA hybridization and PCR amplification (Figures 1A–1C). Two independent ES cell clones carrying the expected recombination event were aggregated with CD1 morulae or microinjected into C57BL/6J blastocysts to generate *Cdk6*$^{+/-}$ mice. Heterozygous mice developed normally and did not show obvious abnormalities. Matings between these mice yielded homozygous *Cdk6*$^{-/-}$ animals at the expected Mendelian ratio (79/279 [28%] *Cdk6*$^{-/-}$ mice versus 66/279 [24%] *Cdk6*$^{+/+}$ animals). Mice lacking Cdk6 did not display gross anatomical abnormalities or increased mortality for up to two years of life. However, mutant females were slightly smaller than their wild-type littermates (15% lower weight at 12 weeks of age) and about 30% were sterile, a defect likely due to a decreased number of mature follicles (about 20%–50% of those present in wild-type ovaries) (data not shown). Whether these defects, along with the reduced size of Cdk6-null females, are due to a hormonal deficiency remains to be determined.

Defective Hematopoiesis in *Cdk6*$^{-/-}$ Mice

Cdk6 is expressed in most mammalian tissues although it is most abundant in lymphoid organs (Meyerson et al., 1992; Meyerson and Harlow, 1994). As illustrated in Figure 2A, Cdk6 was expressed in wild-type but not in Cdk6-null lymphocytes. In Cdk6-deficient mice, the thymus was reduced in size due to lower cellularity ($0.96 \pm 0.3 \times 10^8$ cells in mutant mice versus $1.7 \pm 0.4 \times$

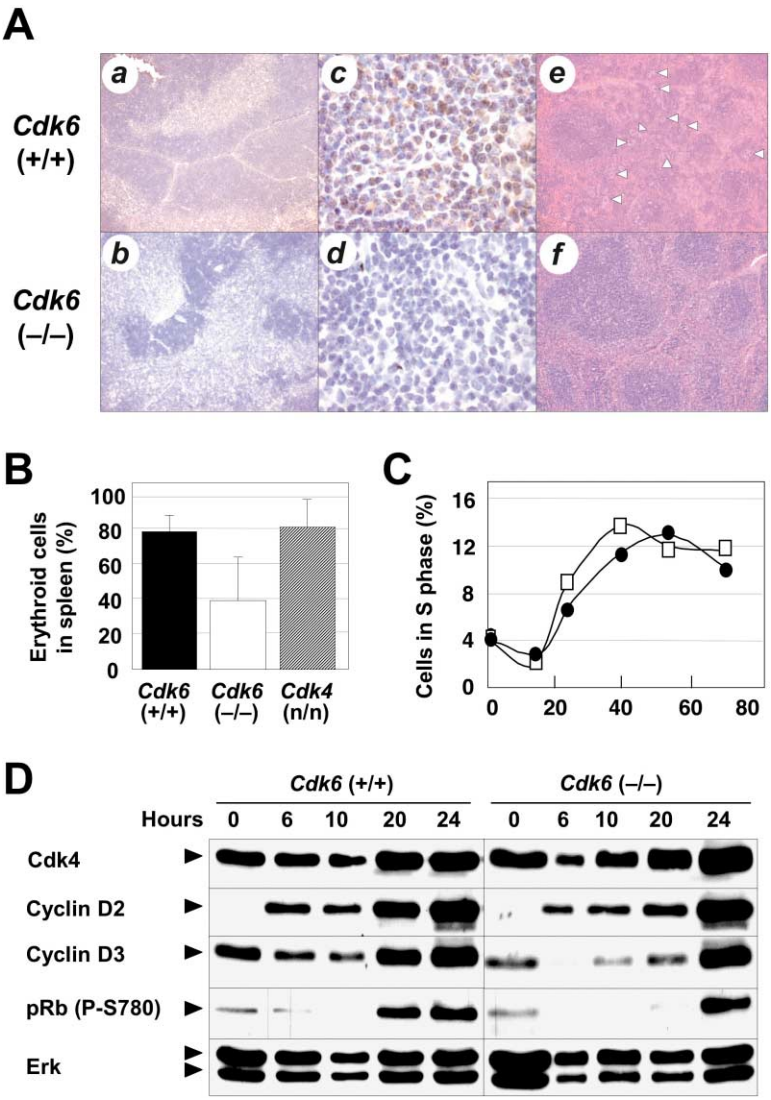

Figure 2. Hematopoietic Defects in *Cdk6*$^{-/-}$ Mice

(A) Immunohistochemical identification of Cdk6 in thymus from (a and c) wild-type and (b and d) and *Cdk6*$^{-/-}$ mice. Representative sections of (e) wild-type and (f) *Cdk6*$^{-/-}$ spleens showing a defect in the red pulp of Cdk6-null mice. Arrowheads indicate megakaryocytes. (a, b, e, and f) 100×; (c and d) 630×.

(B) Percentage of erythroid cells in the spleen of (filled column) *Cdk6*$^{+/+}$, (open column) *Cdk6*$^{-/-}$, and (hatched column) *Cdk4*$^{n/n}$ mice.

(C) Percentage of (open squares) *Cdk6*$^{+/+}$ and (filled circles) *Cdk6*$^{-/-}$ lymphocytes in S phase at the indicated times after stimulation with PMA + Ionomycin.

(D) Expression of cell cycle regulators in lymphocytes from *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice stimulated with PMA + Ionomycin. Cell extracts were analyzed by Western blot at the indicated times using antibodies against Cdk4, cyclin D2, cyclin D3, and Erk and against a phosphorylated serine residue (Ser780) of pRb.

10^8 cells in wild-type littermates, [n = 12]), and its cortical area was atrophic in about one third of the mutant animals (Figures 2Aa and 2Ab). The spleen of *Cdk6*$^{-/-}$ mice was also reduced in size due to decreased cellularity ($1.8 \pm 0.4 \times 10^8$ cells in mutant versus 3.0 ± 0.5 cells in wild-type spleens, [n = 12]). Histological examination revealed normal white pulp but decreased cell density in the red pulp (Figures 2Ae and 2Af). Whereas in wild-type mice 70%–75% of all spleen cells belong to the erythroid lineage, these figures decreased to 35%–40% in *Cdk6*$^{-/-}$ littermates (Figure 2B). Similarly, the number of megakaryocytes was severely reduced to less than one third of those present in wild-type spleens (Figures 2Ae and 2Af).

Red cells in peripheral blood were also decreased in Cdk6-null mice by about 15%. This reduction, however, did not have major physiological consequences since *Cdk6*$^{-/-}$ mice had basically normal levels of hemoglobin in their blood. This compensatory effect is due to an increase in the volume of *Cdk6*$^{-/-}$ erythrocytes and a higher concentration of hemoglobin within red cells (data not shown). The number of granulocytes, macrophages, neutrophils, and platelets was also reduced in the peripheral blood of some, but not all mutant mice (data not shown). The reason for this variability remains to be determined.

Cdk6$^{-/-}$ mice did not display obvious deficiencies in the bone marrow. The medullary space of the femur was filled with densely cellular marrow interspersed with sinusoidal channels as in control mice. Erythroid, myeloid, and lymphoid lineages were represented without maturation gap (data not shown). Medullary fat was also present in the bone marrow of *Cdk6*$^{-/-}$ mice. These observations suggest that Cdk6 is required for expansion of certain differentiated compartments rather than for proliferation of early hematopoietic precursors.

Delayed S Phase Entry in *Cdk6*$^{-/-}$ Lymphocytes

No significant deficiencies were observed in the number of peripheral lymphocytes in *Cdk6*$^{-/-}$ mice. Moreover, B cells and T cells were present at the expected ratios (data not shown). Within thymic T cells, however, there was a slight but consistent increase of CD4 (from 24.5% to 30.6%) and CD8 (from 7.4% to 13.2%) single positive

populations and a consequent decrease of immature double positive cells (from 67.8% to 56.0%). Since Cdk6 is thought to play a key role in the proliferative response of T cells upon mitogenic stimulation, we treated T cells from *Cdk6*$^{-/-}$ mice and control littermates with PMA and ionomycin. Under these conditions, wild-type lymphocytes reached a peak of DNA synthesis in 36–40 hr after mitogenic stimulation. In contrast, the percentage of *Cdk6*$^{-/-}$ lymphocytes in S phase reached maximum levels at 54 hr after treatment (Figure 2C). Similar results were obtained after stimulation with anti-CD3, concanavalin A, or PHA (data not shown). Induction of cyclin D3 expression and, to a lesser extent, cyclin D2 expression were also found to be delayed (Figure 2D). Finally, pRb phosphorylation, a marker of Cdk6 kinase activity, was delayed 10 to 14 hr, at least as determined by phosphorylation of Ser780, a residue thought to be specific for D-type cyclin-dependent Cdks (Figure 2D). These observations provide genetic evidence for the concept that Cdk6 is a regulator of the proliferative response of T lymphocytes upon mitogenic stimuli. Moreover, they illustrate the existence of functional, albeit less efficient compensatory mechanisms that allow proliferation of resting T lymphocytes in the absence of Cdk6.

Unlike *Cdk6*$^{-/-}$ lymphocytes, primary (P2) *Cdk6*-null MEFs grew well in culture and did not display significant proliferative defects (not shown). In addition, serum-starved P2 *Cdk6*$^{-/-}$ MEFs entered S phase with normal kinetics upon serum stimulation. Finally, *Cdk6*$^{-/-}$ MEFs underwent cell culture crisis after a few passages and became immortal upon continuous passage with efficiencies similar to those of wild-type MEFs.

Absence of Synergism between Cdk6 and Cdk2 Deficiencies

Next, we investigated whether concomitant loss of Cdk6 and Cdk2 may have synergistic effects. Double *Cdk6*$^{+/-}$;*Cdk2*$^{+/-}$ heterozygous mice developed normally. Moreover, crosses between these mice yielded all possible genotypes at the expected Mendelian ratios. Mice defective for Cdk6 and Cdk2 display those phenotypical abnormalities previously observed in Cdk2 and Cdk6 single mutants (Ortega et al., 2003; this study). Briefly, *Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ animals are sterile and show marked defects in spermatogenesis and oogenesis, basically indistinguishable to those reported for Cdk2-deficient mice (Ortega et al., 2003). They have limited defects in hematopoietic cells similar to those described above for Cdk6-null mice. Cdk2 and Cdk6 double mutant animals have reduced weight due to a decrease in size similar to that observed in Cdk6 and Cdk2 single mutants. Finally, *Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ animals survive for up to 1.5 years without developing any obvious additional abnormalities.

Late Embryonic Lethality of *Cdk4;Cdk6* Double Mutant Mice

To obtain mice without functional cyclin D-dependent kinases, we crossed *Cdk6*$^{-/-}$ mice with animals heterozygous for a *Cdk4*-null allele, *Cdk4*$^{+/n}$ (Rane et al., 1999). Double heterozygous *Cdk4*$^{+/n}$;*Cdk6*$^{+/-}$ mice developed

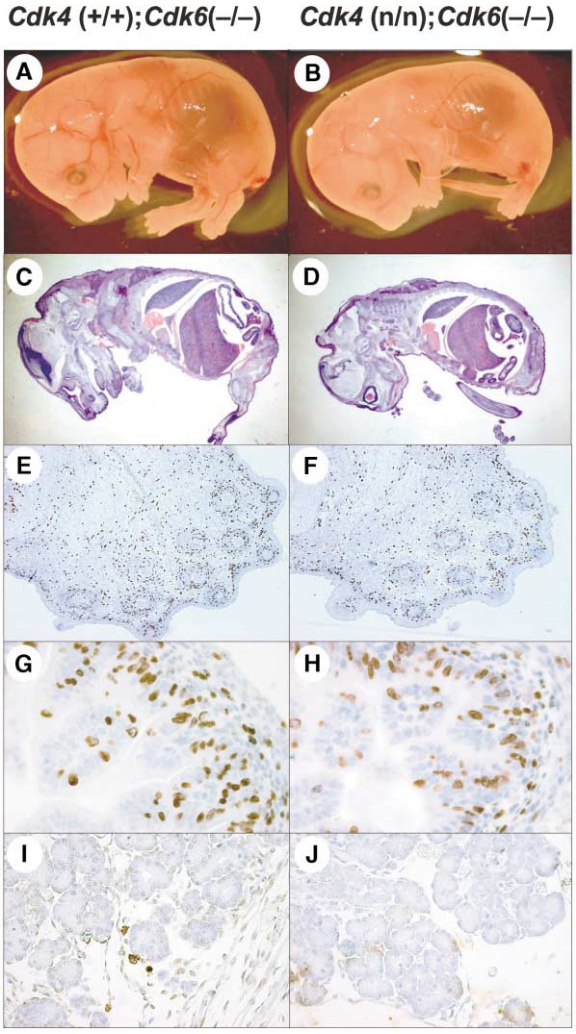

Figure 3. Characterization of Cdk4 and Cdk6 Double Mutant Embryos

E16.5 embryos deficient in (A, C, E, G, and I) Cdk6 or (B, D, F, H, and J) both Cdk4 and Cdk6 display normal organogenesis and cell proliferation in most tissues. (A and B) Wholemount preparation under the binocular microscope; (C and D) Hematoxilin and eosyn staining of a sagital section of the above embryos (16×); BrdU incorporation in (E and F) the skin and whisker follicles (100×) and (G and H) in the intestine (400×); and (I and J) TUNEL assay in the pancreas (100×).

normally and were indistinguishable from wild-type littermates. Crosses between these mice yielded the expected ratio of double heterozygous and *Cdk4*$^{+/n}$;*Cdk6*$^{-/-}$ animals. However, *Cdk4*$^{n/n}$;*Cdk6*$^{+/-}$ mice were born at about one forth the expected ratio and, so far, we have not obtained any live *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ pups.

To determine the reasons for the lack of viability of *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ double knockout mice, embryos derived from crosses between *Cdk4*$^{+/n}$;*Cdk6*$^{-/-}$ mice were collected at various stages of development, ranging from embryonic stages 10.5 to 18.5 (E10.5 to E18.5). E10.5–E12.5 *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos were generated at the expected Mendelian ratio. The number of E14.5–E18.5 embryos was also normal. However, a significant percentage of them were found dead whereas all *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$ embryos were alive. The percentage

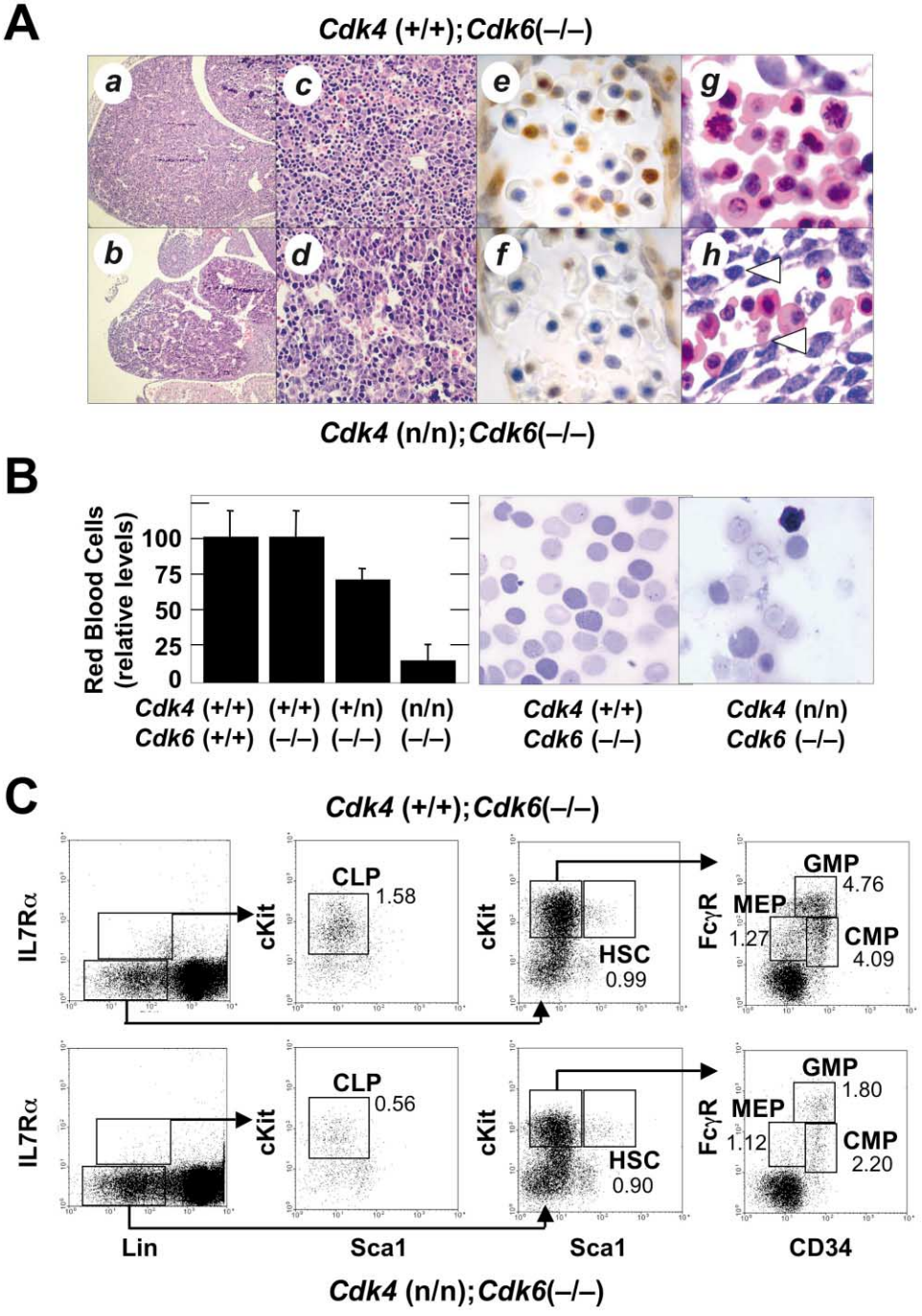

Figure 4. Defective Hematopoiesis in *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ Double Mutant Embryos

(A) E16.5 (a, c, e, g, and i) *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$ and (b, d, f, h, and j) *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos. Liver sections stained with H&E (a and b) 50×; (c and d) 200×. (e and f) Ki67 staining of erythroblasts (1000×); (g and h) blood cells stained with H&E (1000×) showing occasional apoptosis in *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ erythroblasts (arrowheads).

(B) Relative levels of red blood cells in E16.5 embryos of the indicated genotypes. Representative Wright-Giemsa staining of peripheral blood smears is also shown (1000×).

(C) Staining for hematopoietic stem cells (HSC), common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) in fetal liver. The relative percentage of selected cell populations in (top) *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$ and (bottom) *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos is shown.

of dead embryos increased with developmental stage. Whereas at E14.5, only 25% of the *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos were found dead, this percentage increased to 50% at E18.5. A few *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ pups were born. However, all of them were dead when observed a few hours after delivery.

Histological examination of these double null embryos and their corresponding placentas revealed that they

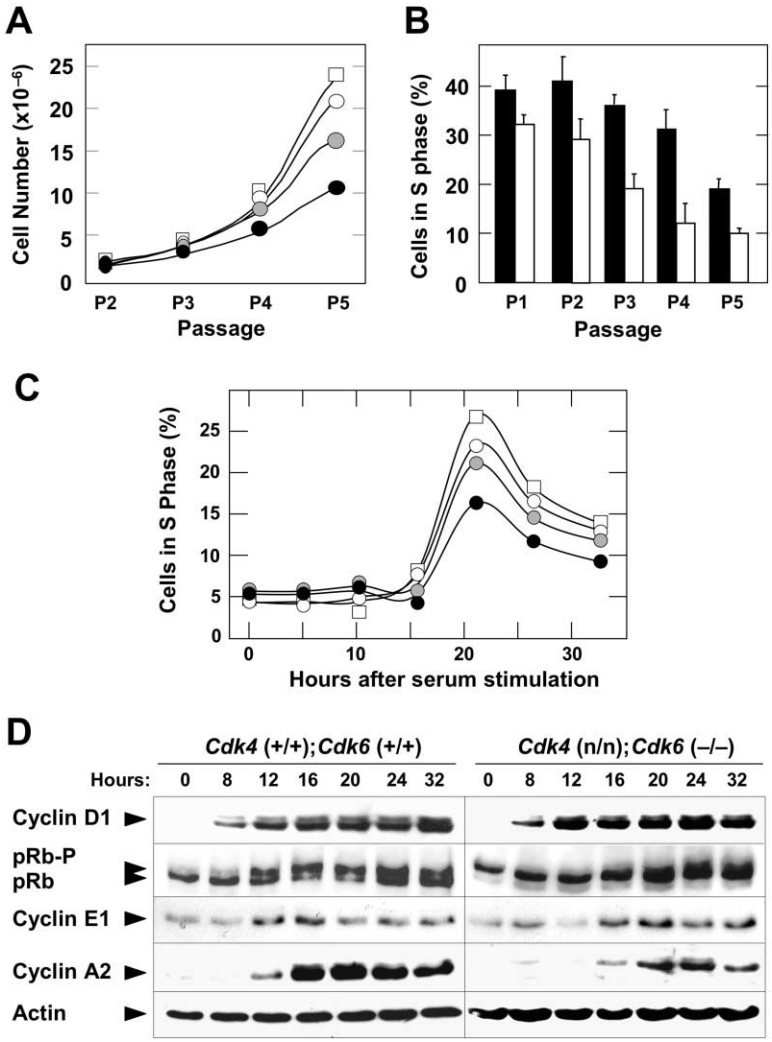

Figure 5. Cell Proliferation in MEFs Lacking Cdk4 and Cdk6

(A) Proliferation of early passage (P2–P5) MEFs derived from (open squares) *Cdk4*$^{+/+}$;*Cdk6*$^{+/+}$; (open circles) *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$; (gray circles) *Cdk4*$^{n/n}$;*Cdk6*$^{+/+}$; and (filled circles) *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos.

(B) Percentage of (filled columns) *Cdk4*$^{+/+}$;*Cdk6*$^{+/+}$ and (open columns) *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs in S phase at the indicated passages (P1 to P5).

(C) Percentage of quiescent MEFs in S phase at the indicated times after serum stimulation. Symbols are those described in (A).

(D) Levels of cyclin D1, phosphorylated and unphosphorylated pRb, cyclin E, cyclin A, and β-actin proteins at the indicated times after serum stimulation of quiescent MEFs derived from *Cdk4*$^{+/+}$;*Cdk6*$^{+/+}$ or *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos.

were well formed and did not present significant abnormalities, except for a consistent reduction in size (about 15% to 20%; Figures 3A–3D). All tissues examined displayed normal levels of Ki67-positive cells (not shown) and BrdU incorporation (Figures 3E–3H). Similarly, these double knockout embryos had normal levels of apoptotic cells in all tissues examined (Figures 3I and 3J). However, starting at developmental stage E14.5, we consistently observed a percentage of dead embryos. E14.5 to E18.5 *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos had structurally aberrant livers that contained decreased levels (40 to 60%) of Ter119+ erythroid precursors (Figures 4Aa–4Ad). This atrophy was associated with dilated and less populated blood vessels. Moreover, less than 2% of these mutant erythroid progenitor cells (erythroblasts) were in a proliferative stage whereas in wild-type or single mutant embryos, 20%–50% of their erythroblasts displayed mitotic figures and were positive for Ki67 (Figures 4Ae and 4Af). We also observed occasional apoptotic figures in erythroblasts of *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ mice but not in littermates with other genotypes (Figures 4Ag and 4Ah).

Analysis of peripheral blood in E16.5–E18.5 *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos revealed a dramatic decrease in the number of red blood cells (Figure 4B). Moreover, these cells depicted a megaloblastic feature typically seen in anemia caused by impaired division of erythroid precursors (Figure 4B). These findings suggest that *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos die during the late stages of embryonic development due to limited proliferation of erythroid progenitors. Interestingly, this phenotype is highly reminiscent of the profound anemia described in embryos deficient for cyclin D2 and cyclin D3, the two D-type cyclins known to be expressed in hematopoietic cells (Ciemerych et al., 2002).

To understand the origin of these hematopoietic abnormalities, we analyzed different hematopoietic stem cells (HSC) and progenitors in fetal livers. By E15.5, *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ livers display a significant reduction in cellularity (7.1×10^6 cells versus 20–22 $\times$ 10^6 cells in *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$ and wild-type livers). The relative levels of double mutant HSCs were not reduced although their absolute numbers were decreased due to the lower cellularity of the mutant livers (Figure 4C). However, the levels of other Cdk4/6-null lineage-committed progenitors, such as common lymphoid progenitors (CLP), common myeloid progenitors (CMP), and granulocyte-macrophage progenitors (GMP), were reduced beyond those

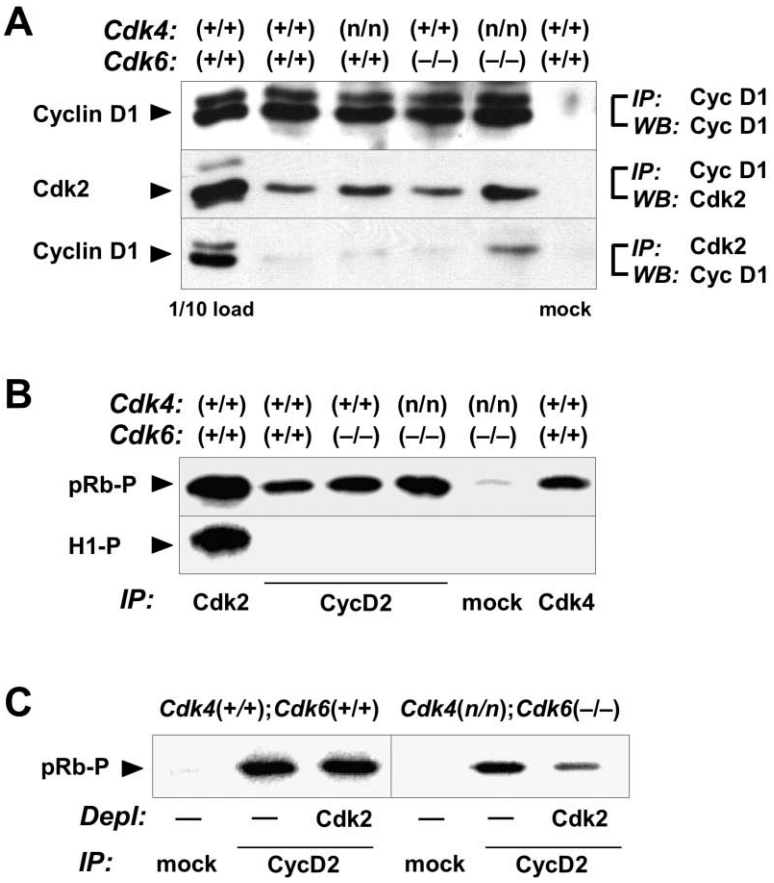

Figure 6. D-Type Cyclin/Cdk Complexes in MEFs Lacking Cdk4 and Cdk6

(A) Identification of cyclin D1/Cdk2 complexes in MEFs derived from embryos with the indicated genotypes. The migration of cyclin D1 and Cdk2 is indicated by arrowheads. One tenth of the total cell extract used for immunoprecipitation was loaded as a control. Another control using an unrelated antibody for immunoprecipitation is also included (mock).

(B) In vitro kinase activity of cyclin D2/Cdk2 immunoprecipitates obtained from *Cdk4*$^{+/+}$;*Cdk6*$^{+/+}$, *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$, and *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs using a fragment of pRb and histone H1 as substrates. Kinase activity of immunoprecipitates obtained from wild-type MEFs with antisera raised against Cdk2 and Cdk4 are included as controls.

(C) In vitro kinase activity in cyclin D2 immunoprecipitates from MEFs derived from *Cdk4*$^{+/+}$;*Cdk6*$^{+/+}$ and *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ embryos after immunodepletion of Cdk2 with anti-Cdk2 antibodies. IP, immunoprecipitation; WB, Western blot; Depl., immunodepletion. Mock indicates immunoprecipitation with an unrelated antibody.

levels expected from the decreased cellularity (Figure 4C). Indeed, the absolute numbers of double mutant CLPs, CMPs, and GMPs were around 15% of those of control livers. Accordingly, the numbers of more mature hematopoietic cells such as monocytes, macrophages, or lymphocytes were also reduced (data not shown). Surprisingly, the reduction of double mutant megakaryocyte-erythroid progenitors (MEP) was less severe (about 40% of controls). Whether this is due to a feedback mechanism that favors the formation of these intermediate precursors in an attempt to compensate for the anemia observed in these double mutant embryos remains to be determined. Finally, a dramatic defect in the proliferative potential of all these precursors was observed using fetal liver cell preparations for functional colony formation assays in methylcellulose. The number of colony-forming units for granulocytes, macrophages, mixed granulocytes/macrophages, and mixed granulocytes/macrophages/erythrocytes/megakaryocytes was severely reduced in *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ livers (less than 5% of controls), whereas the number of erythroid burst-forming units was about 10% of control livers.

MEFs Cycle and Exit Quiescence without Cdk4 and Cdk6

Early passage *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs proliferate well albeit at a slower rate than control cells (Figure 5A). Whereas the doubling time of P2 double mutant MEFs was similar to that of wild-type MEFs (about 28 hr), P4 *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs required a considerably longer time for cell doubling (72 versus 52 hr). Moreover, the percentage of *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs progressing through S phase dropped from 28% at P2 to 14% at P4, a drop not observed in wild-type MEFs (39% versus 36%) (Figure 5B). Finally, at P4–P5, a time when control cells were still proliferating actively, double mutant MEFs begin to display features of senescent cells. Yet, all *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs became immortalized upon continuous passage in culture following a classical 3T3 protocol. For reasons unknown to us, the timing at which these cultures became immortal varied significantly from embryo to embryo. In addition, *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs were also able to proliferate in low serum conditions (2%–5% fetal calf serum) and respond to mitogenic stimuli induced by addition of EGF and IGF-1. However, the rate of proliferation was consistently lower than that observed in control cultures (data not shown). These observations, taken together, indicate that D-type cyclin-dependent kinases are required but not essential for proliferation of embryonic fibroblasts.

Since Cdk4 and Cdk6 complexes have been proposed to be the major sensors for integrating mitogenic signaling during exit from quiescence, we analyzed the response of quiescent *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs to serum stimulation. As illustrated in Figure 5C, serum-starved P2 *Cdk4*$^{n/n}$;*Cdk6*$^{-/-}$ MEFs enter S phase with kinetics similar to those of wild-type MEFs. However, the percentage of cells progressing through the S phase at a given time was only half of that in normal MEFs (Figure

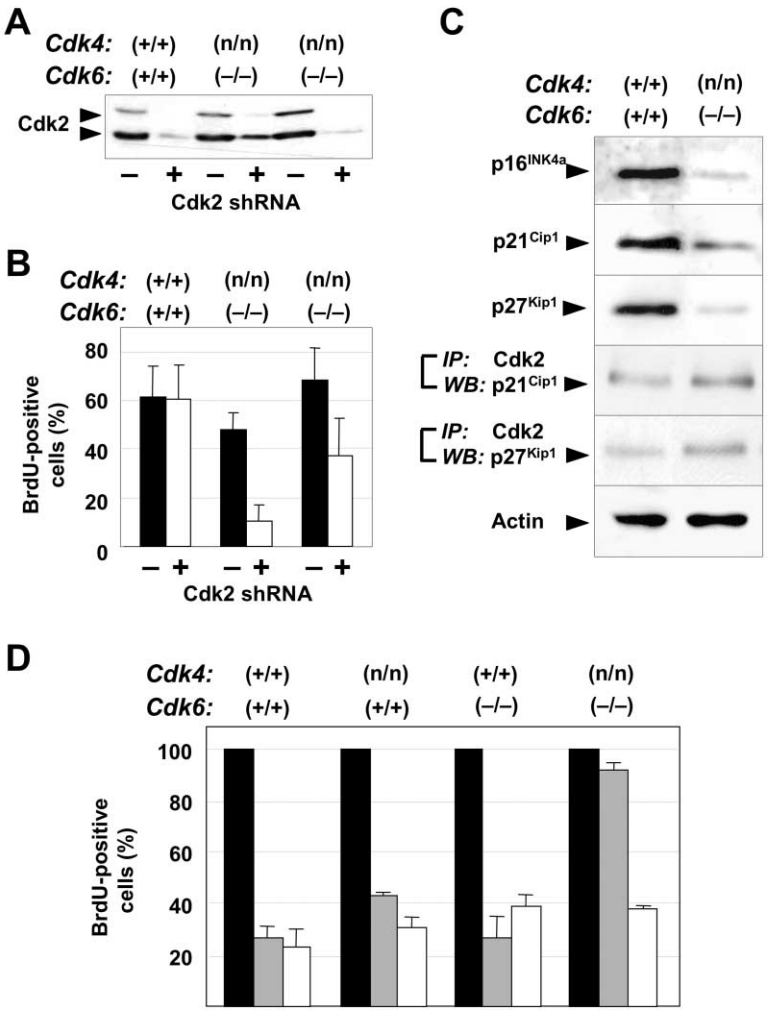

Figure 7. Cell Cycle Arrest by Cdk2 Interference or Cell Cycle Inhibitors in Cdk4/Cdk6-Deficient MEFs

(A) Empty retroviruses (−) or retroviruses expressing a shRNA against Cdk2 (+) were used to infect wild-type or two independent Cdk4 and Cdk6 double mutant MEFs. The migration of the long and short forms of Cdk2 is indicated by arrowheads.

(B) BrdU incorporation in wild-type or Cdk4 and Cdk6 double mutant MEFs infected with (filled columns) empty (−) or (open columns) Cdk2 shRNA (+) retroviruses.

(C) Levels of expression of $p16^{INK4a}$, $p21^{Cip1}$, and $p27^{Kip1}$ cell cycle inhibitors in $Cdk4^{+/+}$; $Cdk6^{+/+}$ and $Cdk4^{n/n}$;$Cdk6^{-/-}$ MEFs. The levels of $p21^{Cip1}$ and $p27^{Kip1}$ complexed with Cdk2 were also determined by immunoprecipitation of Cdk2 with specific antibodies. The migration of these inhibitors and control actin protein is indicated by arrowheads.

(D) Relative levels of BrdU incorporation in MEFs with the indicated genotypes after infection with retroviruses encoding (gray columns) $p16^{INK4a}$ or (open columns) $p21^{Cip1}$ inhibitors with respect to MEFs infected with (filled columns) the retroviral vector alone.

5C). Moreover, primary $Cdk4^{n/n}$;$Cdk6^{-/-}$ MEFs displayed a delay in overall pRb phosphorylation (Figure 5D) as well as in phosphorylation of specific residues such as Ser780 and Ser807/811 (not shown). In agreement with these observations, expression of cyclin E and cyclin A was concomitantly delayed (about 4 hr) in these double mutant MEFs (Figure 5D).

Ablation of Cdk4 and Cdk6 does not affect the expression of D-type cyclins (Figure 5D). Therefore, we examined whether these cyclins could be bound to other partners in the absence of their cognate kinases. Indeed, previous studies have shown that a fraction of cyclin D1 is bound to Cdk2, at least in certain cells (Matsushime et al., 1992; Xiong et al., 1992; Lukas et al., 1995). As illustrated in Figure 6A, D-type cyclins can complex with Cdk2 in wild-type MEFs. These complexes appear to be more abundant in $Cdk4^{n/n}$;$Cdk6^{-/-}$ mutant MEFs. To analyze whether these D-type cyclin/Cdk2 complexes had kinase activity, we immunoprecipitated protein lysates derived from double mutant MEFs with anti-cyclin D2 antibodies (Figure 6B). These immunoprecipitates phosphorylated pRb but not histone H1, a phosphorylation pattern characteristic of D-type cyclin/Cdk4 and Cdk6 complexes. As expected, control Cdk2 immunoprecipitates phosphorylated both pRb and histone H1 (Figure 6B). In $Cdk4^{n/n}$;$Cdk6^{-/-}$ mutant MEFs, pRb kinase activity by cyclin D immunoprecipitates is at least partially due to Cdk2 since immunodepletion with Cdk2 antibodies decreased the levels of pRb phoshorylation (Figure 6C).

To further analyze the possible role of Cdk2 in the proliferation of $Cdk4^{n/n}$;$Cdk6^{-/-}$ MEFs, we infected wild-type and double mutant cells with retroviruses encoding a shRNA against Cdk2. These viruses reduced the amount of Cdk2 protein by 70%–90% (Figure 7A). As illustrated in Figure 7B, Cdk2 shRNA did not affect proliferation of wild-type cells but produced a significant decrease in the rate of BrdU incorporation in $Cdk4^{n/n}$; $Cdk6^{-/-}$ MEFs. These observations suggest that in the absence of Cdk4 and Cdk6, Cdk2 associates with D-type cyclins inducing pRb phosphorylation and cell proliferation.

We have also examined the levels of Cdk inhibitors in cells lacking Cdk4 and Cdk6. As shown in Figure 7C, $Cdk4^{n/n}$;$Cdk6^{-/-}$ MEFs have reduced levels of $p16^{INK4a}$, $p21^{Cip1}$, and $p27^{Kip1}$ inhibitors. These observations could be due to decreased protein stability or to a feedback mechanism that reduces the overall levels of cell cycle inhibitors in the absence of Cdk4 and Cdk6. To distinguish between these two possibilities, we analyzed the

levels of $p21^{Cip1}$ and $p27^{Kip1}$ bound to Cdk2. As illustrated in Figure 7C, Cdk2 immunoprecipitates contained the same levels of these inhibitors as in control MEFs, suggesting that the differences observed in their overall levels are due to the fraction bound to Cdk4 and Cdk6, which is missing in these double mutant cells.

Finally, we investigated whether $Cdk4^{n/n}$;$Cdk6^{-/-}$ cells were still sensitive to the inhibitory properties of $p16^{INK4a}$ and $p21^{Cip1}$. To this end, we infected wild-type, $Cdk4^{n/n}$; $Cdk6^{+/+}$, $Cdk4^{+/+}$;$Cdk6^{-/-}$, and $Cdk4^{n/n}$;$Cdk6^{-/-}$ mutant MEFs with retroviruses encoding either $p16^{INK4a}$ or $p21^{Cip1}$. Whereas $p16^{INK4a}$ strongly inhibited DNA replication (measured as BrdU incorporation) in 60%–80% of wild-type and single mutant MEFs, it failed to block cell cycle progression in $Cdk4^{n/n}$;$Cdk6^{-/-}$ double mutant cells (Figure 7D). In contrast, $p21^{Cip1}$ was able to block BrdU incorporation in all cells. These observations provide genetic evidence for the specificity of $p16^{INK4a}$ in the inhibition of D-type cyclin-dependent kinases, Cdk4 and Cdk6.

Discussion

Cdk4 and Cdk6 are closely related proteins with basically indistinguishable biochemical properties. Thus, the specific roles of these catalytic subunits are most likely determined by their differential patterns of expression. Since Cdk6 is preferentially expressed in hematopoietic cells, it has been proposed as the kinase that initiates pRB phosphorylation in T lymphocytes (Meyerson and Harlow, 1994). Likewise, the differential expression of Cdk4 and Cdk6 has led to the proposal that they regulate cell division at different stages of erythroid maturation (Matushansky et al., 2000). In MEL erythroleukemia cells—transformed erythroid precursor cells blocked at the proerythroblast stage—differentiation requires inhibition of Cdk6 but not of Cdk4 (Matushansky et al., 2000). Accordingly, erythropoietin-dependent terminal differentiation of primary erythroblasts correlates with an early decline of Cdk6 activity followed by a later decline in Cdk4 activity (reviewed in Steinman [2002]).

Our observations indicating that loss of Cdk6 primarily affects the hematopoietic system are, for the most part, in agreement with these observations. For instance, loss of Cdk6 leads to delayed G1 progression in lymphocytes but not in MEFs. In vivo, Cdk6 deficiency results in lower cellularity in hematopoietic tissues such as thymus and spleen. Peripheral blood of Cdk6-deficient mice also had reduced numbers of red blood cells. These observations suggest that Cdk6 is required for expansion of differentiated populations rather than for proliferation of early hematopoietic precursors. Whereas these observations underscore the need of Cdk6 for proper hematopoietic homeostasis, they also indicate that Cdk6 is not essential for proliferation of any specific cell lineage.

Previous genetic studies in *C. elegans* and *D. melanogaster* cyclin D-Cdk4 homologs have shown that these proteins are primarily involved in the regulation of postembryonic growth in specific cell types since ablation of the corresponding genes is compatible with embryonic development (Park and Krause, 1999; Meyer et al., 2000). In contrast, mice lacking Cdk4 and Cdk6 are not viable. Yet, these embryos die during the late stages of embryonic development and display normal cell proliferation and apoptosis in most cell types. Instead, the main defects observed in these double mutant embryos are limited to the compromised maturation of different hematopoietic lineages and severely reduced numbers (about 15% of normal levels) of circulating red blood cells. These defects, which lead to severe anemic conditions, are most likely to be responsible for the death of these double mutant embryos. These observations, taken together, indicate that D-type cyclin-dependent kinases are not essential for proliferation of most cell types, at least during embryonic development. Since these proteins are thought to play a key role in bringing quiescent cells back to the cycle upon mitogenic stimuli, it could be argued that during embryonic development, cells do not withdraw from the cycle or, if they do, they withdraw to a resting state fundamentally distinct from the quiescence state characteristic of nonproliferating adult cells.

These arguments, however, cannot be substantiated by in vitro studies. Cdk4- and Cdk6-deficient MEFs proliferate well, undergo senescence, and become immortal upon continuous passage in a fashion very similar to wild-type MEFs. More importantly, these mutant MEFs become quiescent upon serum withdrawal and re-enter the cycle upon mitogenic stimulation with normal kinetics. Interestingly, these cells display reduced levels of pRb phosphorylation and delayed expression of cyclins E and A, the activating partners of Cdk2. These observations argue against the commonly accepted hypothesis that couples activation of Cdk2 with the start of DNA synthesis. Moreover, they also challenge the widely held concept that full pRb phosphorylation by both Cdk4/6 and Cdk2 kinases is required for proper G1/S transition (Lundberg and Weinberg, 1998; Harbour et al., 1999). Additional studies involving a more detailed characterization of pRb and other transcriptional regulators in these Cdk4/Cdk6 double mutant MEFs will be required to understand the precise role of D-type cyclin/Cdk complexes in cell cycle progression.

Our results establish that D-type-dependent Cdk4 and Cdk6 are not essential for exit from quiescence or for cell cycle progression. Yet, the percentage of MEFs lacking Cdk4 and Cdk6 that re-enter the cycle at a given time upon serum stimulation is about half of those expressing one or both Cdks. Thus, whatever mechanisms may be responsible for compensating the lack of these Cdks are not as efficient in rescuing cells from quiescence. A molecule possibly responsible for this compensatory activity is Cdk2. In agreement with previous reports (Matsushime et al., 1992; Xiong et al., 1992; Meyerson and Harlow, 1994; Lukas et al., 1995; Sweeney et al., 1997), we have observed the association of Cdk2 with D-type cyclins. These complexes are more abundant in the absence of Cdk4 and Cdk6 and, more importantly, have pRb kinase activity. The specificity of this kinase corresponds to that of D-type cyclin/Cdk4 or Cdk6 rather than to E-type cyclin/Cdk2 complexes, an observation consistent with the concept that substrate specificity of Cdks is primarily determined by their partner cyclins (Pavletich, 1999). Moreover shRNA specific for *Cdk2* inhibits proliferation of double mutant but not wild-type MEFs. These results implicate Cdk2 in the proliferation of Cdk4- and Cdk6-deficient cells. However, our observations do not prove that Cdk2 fully compensates

the lack of these Cdks since other kinases might be present in cyclin D immunoprecipitates. Likewise, the used Cdk2 shRNA may also affect expression of other related proteins. In an attempt to resolve this issue, we have undertaken the task of generating triple Cdk4, Cdk6, Cdk2 knockout mice. Unfortunately, loci encoding Cdk4 and Cdk2 map too closely within the mouse genome to allow in vivo recombination at acceptable frequency. Thus, generation of triple recombinant mice requires targeting the *Cdk4* locus within a Cdk2-null background.

Regardless of the outcome of these results, it is unlikely that Cdk2 is sufficient to compensate for the absence of Cdk4 and Cdk6, at least by a mechanism involving interaction with D-type cyclins. In an accompanying manuscript, Sicinski and coworkers (Kozar et al., 2004 [this issue of *Cell*]) report that MEFs lacking the three D-type cyclins grow well in culture and can be rescued from quiescence by mitogenic stimulation with kinetics similar to those observed in Cdk4/Cdk6-deficient MEFs. Thus, it is unlikely that whatever mechanisms are responsible for compensating the lack of Cdk4 and Cdk6 involve D-type cyclins. However, D-type cyclins deficient mice die a couple of days earlier than those lacking their cognate Cdk4 and Cdk6 catalytic subunits. Whereas these subtle differences might be due, at least in part, to different genetic backgrounds, it is also possible that the limited kinase activity provided by D-type cyclin/Cdk2 complexes in Cdk4/Cdk6-null cells may account for the longer life span of the Cdk4/Cdk6 double mutant embryos.

It has recently been reported that Cdk3/cyclin C complexes promote G0/G1 transition in a pRb-dependent manner in several different human cell lines (Ren and Rollins, 2004). This kinase, however, is not active in most laboratory mouse strains (Ye et al., 2001), including those used in this study (our unpublished observations). Therefore, Cdk3 cannot contribute to the observed levels of cell proliferation in the absence of Cdk4 and Cdk6. Whether loss of these kinases in a strain that carries a functional Cdk3 may display a less severe phenotype remains to be determined.

Taken together, our studies, along with those of Kozar et al. (2004), challenge the concept that D-type cyclin/Cdk4 and Cdk6 complexes are the only sensors of mitogenic signaling to rescue cells from quiescence. These findings are reminiscent of recent observations that Cdk2 (Ortega et al., 2003; Berthet et al., 2003) and E-type cyclins (Geng et al., 2003; Parisi et al., 2003) are also dispensable for mitotic cell division. Conditional Cdk4/Cdk6 double mutant mice or triple cyclin D mutant animals will provide additional tools to dissect the role of D-type cyclin/Cdk kinases in mammalian postnatal development and adult homeostasis.

Experimental Procedures

Gene Targeting of *Cdk6*

Mouse *Cdk6* cDNAs and genomic sequences were cloned by plate hybridizations from a mouse testis cDNA library and a lambda genomic library, respectively. For excision of the first exon of Cdk6, which contains the PLSTIRE sequences, we subcloned upstream and downstream genomic fragments in the pPNT vector (Tybulewicz et al., 1991) (Figure 1). The resulting targeting vector, pMM450, was linearized and electroporated into mouse R1 ES cells, and recombinant clones were selected in the presence of G418 and gancyclovir. Seven $G418^R/Gan^R$ clones were identified by Southern hybridization using probes external to the targeting vector (Figure 1). Since the mouse genome contains several Cdk6 genomic pseudogenes (data not shown), those recombinant clones (ESMM1-76 and ESMM1-332) used to generate chimeric mice were further characterized by sequencing of adjacent regions after long-template PCR amplification. These clones were aggregated to CD1 or microinjected into C57BL/6J blastocysts to generate chimeras. No significant differences have been observed between mice derived from these independent clones in 129 × CD1 or 129 × C57BL6/J genetic backgrounds. These heterozygous mice were intercrossed to generate mouse colonies in the 129 × CD1 background or backcrossed to generate a pure C57BL/6J colony. Most of the experiments described in this manuscript were carried out with animals in the 129 × CD1 background. Mice were maintained according to the animal care standards established by the Europan Union. Genotyping of Cdk6-null mice was carried out by PCR amplification using specific oligonucleotides for the normal or mutant allele (primer sequences are available from the authors upon request). Cdk4 and Cdk2 mutant mice were genotyped as previously described (Rane et al., 1999; Ortega et al., 2003).

Histopathologic Analyses

Embryos or organs dissected from mice were fixed in 10%-buffered formalin (Sigma) and embedded in paraffin. Three or five micrometer-thick sections were stained with hematoxylin and eosin. Peripheral blood was obtained from the eye of 2- to 4-month-old mice or from carotid arteries of embryos. Blood smears were prepared using the wedge technique, followed by air-drying and Wright-Giemsa staining. Blood populations were quantified using a blood analyzer (Diatron Messtechnik, Wien) and a flow cytometer (see below).

Immunohistochemistry and Cytometry

For proliferation studies, tissues or embryos were stained with Ki67-specific antibodies (Dako) or labeled with BrdU (Sigma). Pregnant females were injected intraperitoneally with BrdU at 100 μg/g of body weight and embryos and placentas were collected after 2 hr and fixed in formalin. Paraffin sections were stained with anti-BrdU antibody (Becton Dickinson), followed by detection with the Vectastain ABC kit (Vector Laboratories). Detection of apoptotic cells on tissue sections was carried out using the TUNEL assay (Apoptag Peroxidase, Intergen). Cell surface markers used to identify the specific hematopoietic populations in thymus, spleen, bone marrow, or peripheral blood include CD3, CD4, CD8, CD11B, CD25, CD34, CD44, CD69, IL-7, IgM, GR1, Ter119, B220, and F4/80 (Pharmingen). The hematopoietic progenitors in fetal liver were stained as previously described (Akashi et al., 2000; Traver et al., 2001). HSCs were identified as Lin^- $IL7R\alpha^-$ $c\text{-}Kit^+$ $Sca1^+$; CLPs as Lin^- $IL7R\alpha^+$ $c\text{-}Kit^{low}$ $Sca1^{low}$; CMPs as Lin^- $IL7R\alpha^-$ $c\text{-}Kit^+$ $Sca1^-$ $Fc\gamma R^{low}$ $CD34^+$; GMPs as Lin^- $IL7R\alpha^-$ $c\text{-}Kit^+$ $Sca1^-$ $Fc\gamma R^{hi}$ $CD34^+$; and MEPs as Lin^- $IL7R\alpha^-$ $c\text{-}Kit^+$ $Sca1^-$ $Fc\gamma R^{low}$ $CD34^-$. Their relative numbers were quantified by using Coulter (XL) or BD Biosciences cytometers.

Cell Culture Assays

Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS) or 10% calf serum (CS). MEFs were maintained in culture using a standard 3T3 protocol. Proliferation and immortalization assays were performed as previously described (Sotillo et al., 2001). To analyze S phase entry, passage 2 MEFs (10^6 cells per 10 cm dish) were deprived of serum for 48 hr in DMEM + 0.1% FBS and restimulated with 10% FBS to enter the cell cycle. DNA content was analyzed by flow cytometry after propidium iodide staining (Coulter XL or FACScan from Becton-Dickison). Retroviral infection of primary MEFs was performed using pBabe-puro-$p16^{INK4a}$, pBabe-puro-$p21^{Cip1}$, or the empty vector. After infections, cells were selected in puromycin for 2 days and stained for BrdU incorporation after 12 hr in the presence of 50 μM BrdU. For *Cdk2* knockdown experiments, primary MEFs were infected with Cdk2-C, a retroviral construct expressing a shRNA against the

murine *Cdk2* (a gift of P. Sicinski). Primary lymphocytes were isolated from spleen or thymus of 3-month-old mice and cultured in RPMI + 10% FBS. For proliferation studies, T cells were stimulated with PMA and ionomycin, concanavalin A or PHA (Sigma), or anti-CD3 (Pharmingen), and cell cycle profiles analyzed by cytometry. For hematopoietic colony formation assays, fetal livers were collected from E15.5 embryos, and 2–6 $\times$ 10^4 cells were plated in duplicates in methylcellulose supplemented with stem cell factor, interleukin (IL)3, IL6, and erythropoietin (Stem Cell Technologies). Colonies were scored at day 12 as indicated by the manufacturer.

Protein Analysis

Protein lysates were isolated and used for protein analysis by immunoblotting as previously described (Sotillo et al., 2001). Antibodies against the following proteins were used: Cdk2, Cdk1, Cdk4, cyclin A, cyclin E, cyclin D2, $p16^{INK4a}$, $p21^{Cip1}$, Erk (all from Santa Cruz Biotechnology), cyclin D1, (Neo Markers), $p27^{Kip1}$ (Transduction Laboratories), β-actin (Sigma), P-Rb-S780, P-Rb-S795 and P-Rb807/811 (Cell Signaling), and P-Rb-T821 (Biosource International). For kinase assays, 1 μg of mouse pRb protein fragment (amino acids 769–921; Santa Cruz Biotechnology) and histone H1 from calf thymus (Roche Biochemicals) were used as substrates.

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References

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193–197.

Amanatullah, D.F., Zafonte, B.T., Albanese, C., Fu, M., Messiers, C., Hassell, J., and Pestell, R.G. (2001). Ras regulation of cyclin D1 promoter. Methods Enzymol. *333*, 116–127.

Berthet, C., Aleem, E., Coppola, V., Tessarollo, L., and Kaldis, P. (2003). Cdk2 knockout mice are viable. Curr. Biol. *13*, 1775–1785.

Ciemerych, M.A., Kenney, A.M., Sicinska, E., Kalaszczynska, I., Bronson, R.T., Rowitch, D.H., Gardner, H., and Sicinski, P. (2002). Development of mice expressing a single D-type cyclin. Genes Dev. *16*, 3277–3289.

Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. Nat. Rev. Cancer *2*, 910–917.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. *9*, 2364–2372.

Geng, Y., Yu, Q., Sicinska, E., Das, M., Schneider, J.E., Bhattacharya, S., Rideout, W.M., Bronson, R.T., Gardner, H., and Sicinski, P. (2003). Cyclin E ablation in the mouse. Cell *114*, 431–443.

Hanahan, D., and Weinberg, R. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Harbour, J.W., and Dean, D.C. (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev. *14*, 2393–2409.

Harbour, J.W., Luo, R.X., Dei Santi, A., Postigo, A.A., and Dean, D.C. (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell *98*, 859–869.

Kozar, K., Ciemerych, M.A., Rebel, V.I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., and Bhattacharya, S., Bronson, R.T., Akashi, K., and Sicinski, P. (2004). Mouse development and cell proliferation in the absence of D-cyclins. Cell *118*, this issue, 477–491.

Lukas, J., Bartkova, J., Welcker, M., Petersen, O.W., Peters, G., Strauss, M., and Bartek, J. (1995). Cyclin D2 is a moderately oscillating nucleoprotein required for G1 phase progression in specific cell types. Oncogene *10*, 2125–2134.

Lundberg, A.S., and Weinberg, R.A. (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol. Cell. Biol. *18*, 753–761.

Malumbres, M., and Barbacid, M. (2001). To cycle or not to cycle: a critical decision in cancer. Nat. Rev. Cancer *1*, 222–231.

Martín, J., Hunt, S.L., Dubus, P., Sotillo, R., Néhmé-Pélluard, F., Magnuson, M.A., Parlow, A.F., Malumbres, M., Ortega, S., and Barbacid, M. (2003). Genetic rescue of Cdk4 null mice restores pancreatic β-cell proliferation but not homeostatic cell number. Oncogene *22*, 5261–5269.

Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.K., Hanks, S.K., Roussel, M.F., and Sherr, C.J. (1992). Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. Cell *71*, 323–334.

Matushansky, I., Radparvar, F., and Skoultchi, A.I. (2000). Reprogramming leukemic cells to terminal differentiation by inhibiting specific cyclin-dependent kinases in G1. Proc. Natl. Acad. Sci. USA *97*, 14317–14322.

Meyer, C.A., Jacobs, H.W., Datar, S.A., Du, W., Edgar, B.A., and Lehner, C.F. (2000). *Drosophila* Cdk4 is required for normal growth and is dispensable for cell cycle progression. EMBO J. *19*, 4533–4542.

Meyerson, M., and Harlow, E. (1994). Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. *14*, 2077–2086.

Meyerson, M., Enders, G., Wu, C.L., Su, L.K., Gorka, C., Nelson, C., Harlow, E., and Kato, J.Y. (1992). A family of human cdc2-related protein kinases. EMBO J. *11*, 2909–2917.

Moons, D.S., Jirawatnotai, S., Parlow, A.F., Gibori, G., Kineman, R.D., and Kiyokawa, H. (2002). Pituitary hypoplasia and lactotroph dysfunction in mice deficient for cyclin-dependent kinase-4. Endocrinology *143*, 3001–3008.

Morris, E.J., and Dyson, N.J. (2001). Retinoblastoma protein partners. Adv. Cancer Res. *82*, 1–54.

Ortega, S., Prieto, I., Odajima, J., Martín, A., Dubus, P., Sotillo, R., Barbero, J.L., Malumbres, M., and Barbacid, M. (2003). Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. Nat. Genet. *35*, 25–31.

Parisi, T., Beck, A.R., Rougier, N., McNeil, T., Lucian, L., Werb, Z., and Amati, B. (2003). Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells. EMBO J. *22*, 4794–4803.

Park, M., and Krause, M.W. (1999). Regulation of postembryonic G(1) cell cycle progression in Caenorhabditis elegans by a cyclin D/CDK-like complex. Development *126*, 4849–4860.

Pavletich, N.P. (1999). Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors. J. Mol. Biol. *287*, 821–828.

Rane, S.G., Dubus, P., Mettus, R.V., Galbreath, E.J., Boden, G., Reddy, E.P., and Barbacid, M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-cell hyperplasia. Nat. Genet. *22*, 44–52.

Ren, S., and Rollins, B.J. (2004). Cyclin C/cdk3 promotes Rb-dependent G0 exit. Cell *117*, 239–251.

Sherr, C.J., and Roberts, J.M. (1999). Cdk inhibitors: positive and

negative regulators of G1-phase progression. Genes Dev. *13*, 1501–1512.

Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowski, C., Yu, Q., Ferrando, A.A., Levin, S.D., Geng, Y., von Boehmer, H., and Sicinski, P. (2003). Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Cancer Cell *4*, 451–461.

Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J., and Weinberg, R.A. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell *82*, 621–630.

Sicinski, P., Donaher, J.L., Geng, Y., Parker, S.B., Gardner, H., Park, M.Y., Robker, R.L., Richards, J.S., McGinnis, L.K., Biggers, J.D., et al. (1996). Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature *384*, 470–474.

Sotillo, R., Dubus, P., Martín, J., de la Cueva, E., Ortega, S., Malumbres, M., and Barbacid, M. (2001). Wide spectrum of tumors in knock in mice carrying a Cdk4 protein insensitive to INK4 inhibitors. EMBO J. *20*, 6637–6647.

Stacey, D., and Kazlauskas, A. (2002). Regulation of Ras signaling by the cell cycle. Curr. Opin. Genet. Dev. *12*, 44–46.

Steinman, R.A. (2002). Cell cycle regulators and hematopoiesis. Oncogene *21*, 3403–3413.

Stevaux, O., and Dyson, N.J. (2002). A revised picture of the E2F transcriptional network and RB function. Curr. Opin. Cell Biol. *14*, 684–691.

Sweeney, K.J., Sarcevic, B., Sutherland, R.L., and Musgrove, E.A. (1997). Cyclin D2 activates Cdk2 in preference to Cdk4 in human breast epithelial cells. Oncogene *14*, 1329–1340.

Traver, D., Miyamoto, T., Christensen, J., Iwasaki-Arai, J., Akashi, K., and Weissman, I.L. (2001). Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. Blood *98*, 627–635.

Tsutsui, T., Hesabi, B., Moons, D.S., Pandolfi, P.P., Hansel, K.S., Koff, A., and Kiyokawa, H. (1999). Targeted disruption of Cdk4 delays cell cycle entry with enhanced p27Kip1 activity. Mol. Cell. Biol. *19*, 7011–7019.

Tybulewicz, V.L., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell *65*, 1153–1163.

Xiong, Y., Zhang, H., and Beach, D. (1992). D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell *71*, 505–514.

Ye, X., Zhu, C., and Harper, J.W. (2001). A premature-termination mutation in the Mus musculus cyclin-dependent kinase 3 gene. Proc. Natl. Acad. Sci. USA *98*, 1682–1686.