Genetic Evidence for Functional Dependency of p18^{Ink4c} on *Cdk4*

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The INK4 family of cyclin-dependent kinase (CDK) inhibitors negatively regulates cyclin D-dependent CDK4 and CDK6 and induces the growth-suppressive function of Rb family proteins. Mutations in the *Cdk4* gene conferring *INK4* resistance are associated with familial and sporadic melanoma in humans and result in a wide spectrum of tumors in mice, suggesting that INK4 is a major regulator of CDK4. Mice lacking the *Cdk4* gene exhibit various defects in many organs associated with hypocellularity, whereas loss of the p18^{Ink4c} gene results in widespread hyperplasia and organomegaly. To genetically test the notion that the function of INK4 is dependent on CDK4, we generated *p18*; *Cdk4* double-mutant mice and examined the organs and tissues which developed abnormalities when either gene is deleted. We show here that, in all organs we have examined, including pituitary, testis, pancreas, kidney, and adrenal gland, hyperproliferative phenotypes associated with *p18* loss were canceled. The double-mutant mice exhibited phenotypes very close to or indistinguishable from that of *Cdk4* single-mutant mice. Mice lacking p27^{Kip1} develop widespread hyperplasia and organomegaly similar to those developed by *p18*-deficient mice. The *p27*; *Cdk4* double-mutant mice, however, displayed phenotypes intermediate between those of *p27* and *Cdk4* single-mutant mice. These results provide genetic evidence that in mice p18^{Ink4c} and p27^{Kip1} mediate the transduction of different cell growth and proliferation signals to CDK4 and that p18^{Ink4c} is functionally dependent on CDK4.

The progression of eukaryotic cells through different phases of mitotic division is controlled primarily by the cyclin-dependent kinase (CDK) whose activity is in turn balanced by its activation by a requisite cyclin subunit and its inhibition by a CDK inhibitor. In mammalian cells, there exist two distinct families of CDK inhibitors. The p21 family includes three related proteins, p21$^{Cip1/Waf1}$, p27^{Kip1}, and p57^{Kip2}, which evolved from a common ancestor that predates *Caenorhabditis elegans* and plants. The p16 family consists of four closely related members, p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}, and evolved later, after the emergence of vertebrates.

The physiologic significance of evolving a separate family of CDK inhibitors and multiple members within each family in mammalian cells is presumed to meet increasing needs for integrating more intricate and multifaceted cell growth signals, both intracellular and extracellular, into a single cell cycle control machinery. Supporting this notion are the observations that the expression of individual CDK inhibitor genes is activated selectively by different checkpoint pathways, displays distinct temporary and spatial patterns during both in vitro cell differentiation and in vivo embryonic development, and is maintained differentially in different adult and senescent tissues. Further support for different physiologic functions of individual CDK inhibitor genes in vivo comes from the genetic analyses of mutant strains of mice with targeted mutation in each of seven individual CDK inhibitor genes. Various different phenotypes were observed in these mice, ranging from lack of major defects after the loss of the p15 or the p19 gene (27, 55), compromised DNA damage response after inactivation of the p21 gene (4, 8), increase of tumor development resulting from loss of function of p16 (25, 45), and severe developmental defects after p57 mutation (54) to widespread hyperplastic cell proliferation and organomegaly in p18 and p27 null mice (14, 15, 23, 27, 37). Elucidation of the molecular pathways linking various cell growth signals to the expression of individual CDK inhibitor genes, however, remains a major challenge and has only been validated in a few exceptional cases. Two examples are the p53-mediated activation of *p21* gene expression following DNA damage (11) and transforming growth factor β-mediated induction of p15^{Ink4b} (17).

Biochemically, CDK inhibitors within each family act almost indistinguishably in binding to and regulating CDK enzymes but differ among the families. The CIP/KIP proteins share a unique N-terminal sequence motif comprising two subdomains for binding cooperatively to and forming ternary complexes with CDK and cyclin subunits. The CIP/KIP proteins diverge in their C-terminal sequences. The INK4 proteins, on the other hand, consist essentially of four or five tandem copies of ankyrin repeats and few additional sequences. Unlike CIP/KIP inhibitors, which are capable of interacting with multiple CDK-cyclin complexes, the only binding partners and functional targets identified thus far for INK4 proteins are two very closely related catalytic CDK subunits, CDK4 and CDK6. Such functional dependency of INK4 on CDK4 or CDK6, however, has not yet been tested genetically.

Ectopic overexpression of individual INK4 genes causes a G$_1$ cell cycle arrest with a correlative dependency on the intact Rb pathway (16), and loss of either Rb function or a combination of p107 and p130 functions effectively canceled the G$_1$

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arrest due to INK4 overexpression (3, 24, 35). These findings provide evidence that, at least in cultured cells, the function of INK4, and CDK4 and CDK6 by extension, in controlling the G_1-to-S transition is dependent on the presence of both an intact Rb and p107-p130 functions. Hence, there may exist in vivo a linear INK4-CDK4/CDK6 (CDK4/6)-Rb G_1 control pathway in mammalian cells. Delineating the pathway(s) for p21 family inhibitors in vivo is more complicated and remains somewhat perplexing; this pathway is attributed largely to their interaction with both the CDK and cyclin subunits and with multiple CDK-cyclin complexes. It was initially observed that p21 levels undergo an increase immediately following mitogenic stimulation of serum-starved human fibroblasts, before declining at the G_1-S boundary (29), and that CDK and cyclin can be found in active CDK-cyclin complexes at a one-to-one ratio when expressed at a low concentration (18, 53). Later studies found that the assembly and kinase activity of CDK4-cyclin D correlate concomitantly with the binding of CIP/KIP proteins (26) and were reduced in mouse embryonic fibroblasts (MEFs) lacking p21 and p27 (6). A titration model—cyclin Ds-CDK4/6 complexes act as activators of cyclin Es-CDK2 complexes by titrating CIP/KIP proteins away from, and thus releasing the inhibition of, cyclin Es-CDK2 complexes—was proposed to accommodate these observations, which seemingly contradict the classification of CIP/KIP as a CDK inhibitor. According to this model, CIP/KIP genes can be considered to act genetically downstream of cyclin Ds-CDK4 complexes in an INK4-cyclin Ds/CDK4-CIP/KIP-cyclin Es/CDK2-Rb pathway. Results challenging both notions—that p21 only stoichiometrically inhibits cyclin A-CDK2 and that p21-p27 deficiency reduced cyclin D-CDK4 assembly and activity—were reported (1, 19), leaving the mechanistic role of CIP/KIP proteins in regulating cyclin Ds-CDK4/6 and cyclin Es-CDK2 complexes at an incompletely understood and somewhat confusing state at present.

The p18^{Ink4c} and p27^{Kip1} genes represent two of the most extensively studied CDK inhibitor genes in mice. Loss of function of either gene resulted in similar and widespread defects in cell proliferation and organ development, providing suitable models for investigating the functional and mechanistic differences between the two families of CDK inhibitors in living animals. In this study, we set forth to test genetically in double-mutant mice the following questions: is the function of INK4 to inhibit CDK4, and how does the loss of *Cdk4* and a gene from either CDK inhibitor family affect each other phenotypically in mice?

MATERIALS AND METHODS

Generation of mouse strains. The generation of *p18* and *Cdk4* mutant mice has been described previously (15, 49). Both have been backcrossed into and maintained on an enriched C57BL/6 background as previously reported (2, 49). Mice deficient for *p18* were bred to the mice heterozygous for *Cdk4* to create double-heterozygous mice. Mice heterozygous for *p18* and *Cdk4* were intercrossed to generate all of the genotypes analyzed in this study. Animals were genotyped by PCR and monitored as described previously (15, 49). Cohorts were housed and analyzed in a common setting, and littermate controls were used for all experiments.

Analysis of fertility and diabetes. Mating ability and fertility of mice were determined by keeping each mutant and a wild-type fertile partner together in a cage and checking daily for the presence of a vaginal plug. Females with plugs were separated and monitored throughout pregnancy, delivery, and nursing. Blood glucose level was monitored in the morning (8 to 10 a.m.) by using an automatic glucose monitor (Glucometer Elite; Bayer). Each mouse was analyzed twice for blood glucose on two consecutive days for every time point.

Histopathology. Tissues of most organs were removed, fixed in 10% neutral buffered formalin, and examined histologically by two pathologists after hematoxylin-eosin staining. Lesions were photographed, and additional sections were taken for immunohistochemical analyses.

Pancreatic islet size determination. Pancreatic islet size (cells per islet section) was measured as previously reported with minor modifications (33). Briefly, islet size was determined in at least three cut sections from matched pancreatic regions of three animals per genotype at each stage of development. Sections were more than 500 μm from each other to avoid overestimating larger islets in this analysis.

Immunohistochemistry. To measure proliferating and mitotic cells, sections were blocked with normal goat serum in phosphate-buffered saline (PBS) and incubated with either a polyclonal antibody against mitosis-specific phosphorylated histone H3 (5 μg ml^{-1}; Upstate Biotechnology) or a polyclonal antibody against Ki67 (1:1,000; NCL-Ki67; Novocastra Laboratories) for 1 h and with a biotin-conjugated secondary antibody (Vector Laboratories) for 30 min. Immunocomplexes were detected with the Vectastain ABC alkaline phosphatase kit according to the manufacturer's instructions (Vector Laboratories). For apoptosis assays, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were carried out using the in situ ApopTag kit (Intergen) according to the manufacturer's protocol.

MEFs and flow cytometry procedures. Primary MEFs were isolated from embryonic day 13.5 embryos. Early-passage MEFs (younger than passage 4) from individual embryos were plated in 100-mm plates and incubated in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS). For serum starvation, asynchronous cultures at approximately 50% confluence were washed with PBS and serum starved in DMEM containing 0.1% FBS for 72 h. Cells were restimulated by addition of DMEM with 10% FBS. For bromodeoxyuridine (BrdU) labeling, MEFs were grown in media with or without serum containing 10 μM BrdU for 1 h. Nuclei were isolated following trypsinization and fixation with 95% ethanol by incubation in 0.08% pepsin (Sigma)–0.1 N HCl for 20 min at 37°C. Nuclear DNA was denatured by incubation in 2 N HCl for 20 min at 37°C, followed by neutralization with Na$_2$B$_4$O$_7$, pH 8.5. Incorporated BrdU was detected with anti-BrdU-fluorescein isothiocyanate (Becton Dickinson; 1:10 dilution) in 10 mM HEPES (pH 7.3)–150 mM NaCl–4% FBS–0.5% Tween 20. MEFs were then harvested and resuspended in PBS containing 1% FBS and incubated with propidium iodide (5 μg ml^{-1}) and RNase A (0.1 mg ml^{-1}) for 30 min at 37°C. The samples were analyzed with a B-D FACScan (Becton Dickinson), and the data were processed with Summit software (version 3.0; BD Biosciences).

Northern blot analysis. MEFs at early passage were serum deprived (0.1% FBS) for 3 days and released from quiescence by serum stimulation (10% FBS). Total RNA was prepared from cells at different time points after serum stimulation as well as from an asynchronized cell population. RNA samples were resolved on a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized with a probe derived from full-length mouse p18^{Ink4c} cDNA. The blot was then stripped and rehybridized with a mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe.

RESULTS

Loss of *Cdk4* prevented gigantism of *p18* mutant mice but only partially rescued gigantism of *p27* mutant mice. Mutant *p18*$^{+/-}$; *Cdk4*$^{+/-}$, *p18*$^{-/-}$; *Cdk4*$^{+/-}$, *p18*$^{+/-}$; *Cdk4*$^{-/-}$, and *p18*$^{-/-}$; *Cdk4*$^{-/-}$ mice were generated from mating double-heterozygote *p18*$^{+/-}$; *Cdk4*$^{+/-}$ mice. *p18*$^{-/-}$; *Cdk4*$^{-/-}$ mice were born with a ratio of 4.4% (8 of 183), lower than their wild-type (9.3%, 17 of 183) and *p18*$^{-/-}$; *Cdk4*$^{+/+}$ (10.4%, 19 of 183) siblings, but comparable to *Cdk4*$^{-/-}$ littermates (3.8%, 7 of 183; Table 1). These results indicate that disruption of *Cdk4* may induce a prenatal lethality with incomplete penetrance as previously reported (40) and that deletion of *p18* has no significant effect on prenatal lethality caused by *Cdk4* deficiency.

At birth, *Cdk4*$^{-/-}$, *p18*$^{-/-}$, and *p18*$^{-/-}$; *Cdk4*$^{-/-}$ double-mutant mice of various genotypes appeared indistinguishable (data not shown). Soon after birth, however, various growth abnormalities were grossly apparent. While the *p18*$^{-/-}$ mice

TABLE 1. Incomplete penetrance of prenatal lethality in *Cdk4* mutant and double-mutant mice[a]

Progeny	No (%) of mice with genotype:								
	$p18^{+/+}$; $Cdk4^{+/+}$	$p18^{+/+}$; $Cdk4^{+/-}$	$p18^{+/+}$; $Cdk4^{-/-}$	$p18^{+/-}$; $Cdk4^{+/+}$	$p18^{+/-}$; $Cdk4^{+/-}$	$p18^{+/-}$; $Cdk4^{-/-}$	$p18^{-/-}$; $Cdk4^{+/+}$	$p18^{-/-}$; $Cdk4^{+/-}$	$p18^{-/-}$; $Cdk4^{-/-}$
Observed	17 (9.3%)	25 (13.7%)	7 (3.8%)	27 (14.8%)	40 (21.9%)	16 (8.7%)	19 (10.4%)	24 (13.1%)	8 (4.4%)
Expected	11 (6.3%)	23 (12.5%)	11 (6.3%)	23 (12.5%)	46 (25%)	23 (12.5%)	11 (6.3%)	23 (12.5%)	11 (6.3%)

[a] Newborn pups were assessed for viability and genotyped. Mice were derived from $p18^{+/-}$; $Cdk4^{+/-}$ intercross (183 pups in 19 litters).

lived up to 1 year, at which time they developed both pituitary tumors and lymphomas, the majority of $Cdk4^{-/-}$ mice died early in life (<4 months) as previously reported (40, 49), but 5 to 10% of $Cdk4^{-/-}$ mice survived up to 1 year without diabetes (our unpublished results). The genetic basis for this incomplete penetrance of the diabetes phenotype resulting from *Cdk4* loss is not clear at present. Loss of *p18* had no significant effect on the postnatal mortality caused by *Cdk4* loss or on the development of double-mutant mice. No $p18^{-/-}$; $Cdk4^{-/-}$ ($n = 9$) or $p18^{+/-}$; $Cdk4^{-/-}$ ($n = 11$) animal lived beyond 5 months.

Growth retardation of $p18^{-/-}$; $Cdk4^{-/-}$ mice became obvious during prepuberty, was persistent through the entire experimental duration of 9 weeks, and was observed in both sexes. The body weights of both $p18^{-/-}$; $Cdk4^{-/-}$ and $p18^{+/-}$; $Cdk4^{-/-}$ mice were lower than those of either $p18^{-/-}$ or wild-type mice and were statistically indistinguishable from those of $Cdk4^{-/-}$ single null mice (Fig. 1A and C). Thus, loss of *Cdk4* led to a body weight phenotype that was completely dominant over loss of *p18*.

Loss of function of the *p27* gene similarly resulted in an increased body weight, as seen in mice with the *p18* mutation (14, 23, 37). To determine how *Cdk4* and *p27* genes interact with each other in controlling the body weight, we analyzed the *p27 Cdk4* double-mutant mice. The $p27^{-/-}$; $Cdk4^{-/-}$ double-mutant mice exhibited a body weight intermediate between those of the single-homozygous mutant mice (Fig. 1B), indicating that loss of *Cdk4* and *p27* partially rescued the body weight abnormality caused by the loss of the other gene. Together, these results demonstrate that, although *p18* and *p27* gene disruption resulted in a wide range of similar phenotypes in mice, these two genes do not have the same mechanism for regulation of *Cdk4* in vivo. While the phenotype caused by *p18* inactivation was completely dependent on CDK4, the function of p27 was only partially dependent on CDK4.

Both male and female $p18^{-/-}$; $Cdk4^{-/-}$ mice are infertile. All $p18^{-/-}$; $Cdk4^{-/-}$ mice, both males ($n = 8$) and females ($n = 10$), that we examined are infertile, and most (more than 90%) of $Cdk4^{-/-}$ males and all female mutants are sterile, as reported previously. In contrast to testes enlargement caused by *p18* loss, the testes of 5-month-old $p18^{-/-}$; $Cdk4^{-/-}$ mice were small and had sizes comparable to those of testes of $Cdk4^{-/-}$ mice (Fig. 2A). Histological analysis revealed that, while $p18^{-/-}$ male mice developed Leydig cell hyperplasia, the $p18^{-/-}$; $Cdk4^{-/-}$ male mouse, like the $Cdk4^{-/-}$ mouse, contains abnormal seminiferous tubules with degeneration of a significant fraction of primary spermatocytes and reduced Leydig cells. Very few normal spermatozoa were found in the lumen of seminiferous tubules of both $Cdk4^{-/-}$ and $p18^{-/-}$; $Cdk4^{-/-}$ mice at an age of 5 months (Fig. 2A). Hence, the seminiferous tubules in mice lacking both *Cdk4* and *p18* genes

exhibited defects similar to those found in $Cdk4^{-/-}$ mice. Leydig cell proliferation associated with p18 loss was inhibited by *Cdk4* loss.

$p18^{-/-}$ females are fertile and can mate successfully with wild-type or $p18^{-/-}$ males (15). Litter numbers and sizes of mice produced from $p18^{-/-}$ matings were bigger than those for age-matched wild-type mating controls (F. Bai and Y. Xiong, unpublished data). $p18^{-/-}$ ovaries are bigger (twice as big as the wild type), displayed normal antral follicles, and formed many corpus lutea (CL). These results suggest that, despite ovarian enlargement, ovulation from $p18^{-/-}$ mice was not impaired. $Cdk4^{-/-}$ female mice, like $p27^{-/-}$ female mice, are infertile. Adult $Cdk4^{-/-}$ ovaries were 20 to 30% smaller than those of wild-type females and showed some well-developed antral follicles but very few CL (at most one CL in each ovary). Very similar to $Cdk4^{-/-}$ ovaries, $p18^{-/-}$; $Cdk4^{-/-}$ ovaries were small and rarely developed CL at 4 months of age, when many CL were found in littermate wild-type and $p18^{-/-}$ control ovaries (Fig. 2), indicating that loss of *p18* does not alleviate the infertility caused by defective luteal function in *Cdk4*-deficient mice.

Loss of *p27* also resulted in the development of ovarian enlargement but impaired the function of ovulation and caused female sterility (14, 23, 37, 42, 47), indicating that p18 and p27 play different roles in ovulation and granulosa cell proliferation. $p27^{-/-}$; $Cdk4^{-/-}$ female (as well as male) mice are infertile.

Development of pituitary hyperplasia in $p18^{-/-}$ mice requires CDK4 function. A hallmark defect in mice with reduced or complete loss of *Rb* function is the hyperplastic phenotype of the pituitary (20, 21, 28, 31, 51). Loss of *p18* results in the development of the same hyperplastic phenotype in the intermediate lobe of the pituitary (15, 27) (Fig. 3A) and, conversely, loss of *Cdk4* causes a pituitary hypoplasia and lactotroph dysfunction (36), offering an excellent in vivo setting to determine the genetic interaction between these two Rb regulators. We confirmed that pituitaries of *Cdk4* null adult mice (4 months old) were significantly smaller and extremely hypoplastic relative to wild-type pituitaries in all three lobes: the intermediate lobe, the anterior lobe, and the neurohypophysis (Fig. 3A). The pituitaries of $p18^{-/-}$; $Cdk4^{-/-}$ mice were as small as *Cdk4* null pituitaries and also showed significant hypoplasia, which might be responsible for the infertility and defect in formation of CL in $p18^{-/-}$; $Cdk4^{-/-}$ mice. Like those from pituitaries of *Cdk4* single-knockout mice, the intermediate lobes from $p18^{-/-}$; $Cdk4^{-/-}$ pituitaries were hypoplastic, indicating that the development of pituitary hyperplasia in $p18^{-/-}$ mice requires a functional CDK4. Late in life (>9 months), $p18^{-/-}$ mice developed pituitary adenomas in the intermediate lobe. Because of early death of $p18^{-/-}$; $Cdk4^{-/-}$ mice, we were not able to

demonstrate that loss of *Cdk4* would prevent the development of pituitary adenomas.

To determine the cellular basis of functional dependency of *p18* on *Cdk4*, we examined two cellular defects, apoptotic cell death and cell proliferation, often associated with an alteration of genes involved in the Rb pathway. Pituitary glands were isolated from mice of different genotypes. Serial sections were examined by TUNEL assay to determine the level of apoptosis, by Ki-67 staining to determine the level of cell proliferations, and by staining with an antibody against phosphorylated histone H3 to determine mitotic index. Apoptosis in any of the three lobes of the wild-type pituitary was nearly undetectable (Fig. 3B). In the $p18^{-/-}$ pituitary, apoptosis was barely detectable in both the intermediate lobe and neurohypophysis and was very low in the anterior lobe (Fig. 3B), indicating that an abnormal hyperproliferation caused by *p18* loss is not associated with an increase of apoptosis and that a net increase of cell number is therefore responsible for the increase in the size of the intermediate lobe. Loss of *Cdk4* and *p18* did not cause a detectable increase of apoptosis in both the intermediate lobe and neurohypophysis. But loss of *Cdk4* substantially increased the apoptotic cell death in the anterior lobe, which might be responsible for defective prolactin production and CL formation. Deletion of the *p18* gene did not detectably reduce the apoptosis caused by *Cdk4* loss in the anterior lobe of the $p18^{-/-}$; $Cdk4^{-/-}$ pituitary (Fig. 3B).

Both Ki-67 and phosphor-H3 staining revealed an increase of cell proliferation in the $p18^{-/-}$ pituitary and a decrease of cell proliferation to almost the basal level in the $Cdk4^{-/-}$ pituitary (Fig. 3B). Increase of both Ki-67- and phosphor-H3 positive cells in the $p18^{-/-}$ pituitary is not restricted to the intermediate lobe; it is also evidently in both the anterior lobe and the neurohypophysis (Fig. 3B), revealing a previously unrecognized function of p18 in these parts of the pituitary. Consistent with these results, we also found that *p18* is expressed in all three lobes of the pituitary (data not shown). As apoptosis in both cell types was not significantly increased, an increase of cell proliferation, but not the size of both anterior lobe and neurohypophysis, suggests that a separate compensating mechanism may maintain an overall normal tissue size in these parts of pituitary. The numbers of both Ki-67- and phosphor-H3-positive cells in the $p18^{-/-}$; $Cdk4^{-/-}$ pituitary were similar to those seen in the wild type, indicating that loss of the *Cdk4* gene inhibited hyperproliferation caused by *p18* loss.

Loss of *p18* does not rescue diabetes of *Cdk4*-deficient mice. *Cdk4*-deficient mice displayed normoglycemia at 3 weeks of age and developed diabetes around 7 weeks of age (40, 49) (Fig. 4A). Histological analysis revealed that by the age of 3 weeks the number and size of pancreatic islets were not affected by the loss of CDK4. However, by the ages of 7 and 16 weeks the number of pancreatic islets was significantly decreased. Pancreases of wild-type mice showed 82.6, 16.6, and 0.8% small, medium, and large islets, respectively (Fig. 4B and C). The number of islets from $Cdk4^{-/-}$ mice was 50% less than the number from wild-type littermate mice (Fig. 4B and data not shown). Analysis of the sizes of the islets found in $Cdk4^{-/-}$ pancreases of mice at 16 weeks of age revealed that 96.8% were small (1 to 40 cells/islet section), that only 3.2% were medium sized (41 to 400 cells/islet section), and that there were no large-sized islets (>400 cells/islet section). These re-
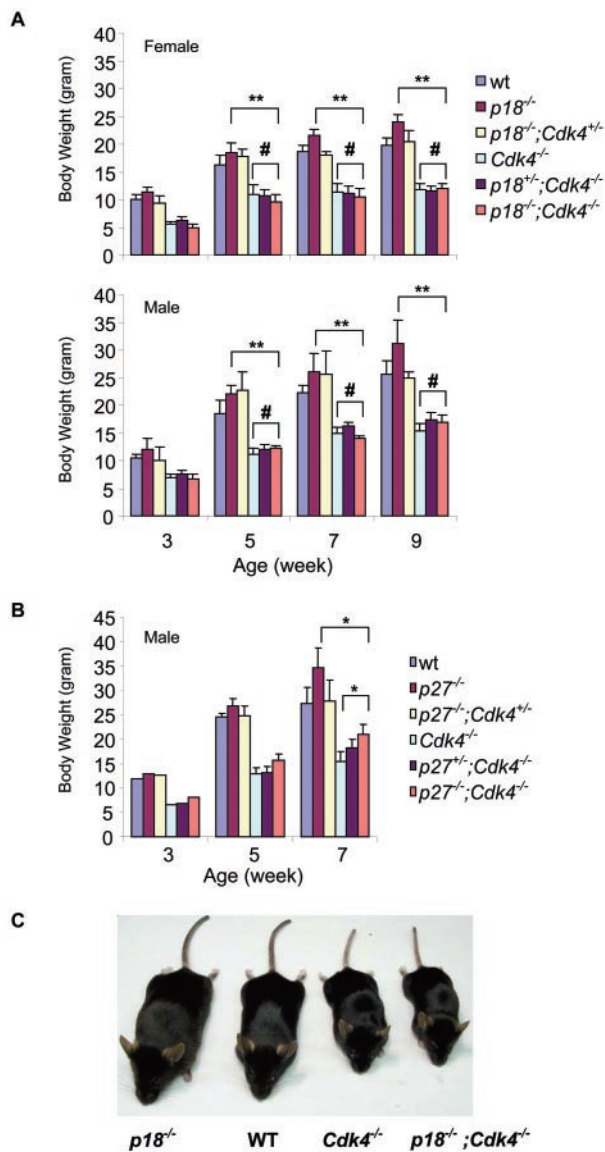

FIG. 1. Effect of the loss of *Cdk4*, *p18*, and p27 on animal growth. (A) Body weight comparison for male and female mice of different genotypes. Mice from every genotype (four to six each) were weighed from age 3 to 9 weeks. Standard deviation bars are indicated. **, $P <$ 0.001, for comparison of $p18^{-/-}$; $Cdk4^{-/-}$ mice to $p18^{-/-}$ mice; #, $P >$ 0.05 (no significant difference), for comparison of $p18^{-/-}$; $Cdk4^{-/-}$ mice to $Cdk4^{-/-}$ mice (Student's *t* test). (B) Body weight comparison of $Cdk4^{-/-}$, $p27^{-/-}$, and $p27^{-/-}$; $Cdk4^{-/-}$ mutant male mice. Mice from each genotype (three or four each) were weighed from age of 3 to 7 weeks. Standard deviation bars are indicated. *, $P < 0.05$, for comparison of $p27^{-/-}$; $Cdk4^{-/-}$ mice to $Cdk4^{-/-}$ or $p27^{-/-}$ mice (Student's *t* test). (C) $p18^{-/-}$, wild-type (WT), $Cdk4^{-/-}$, and $p18^{-/-}$; $Cdk4^{-/-}$ mice at 4 months of age.

sults suggest that not only islet number but also the size of the islets were significantly decreased in *Cdk4*-deficient mice. In contrast, the number of islets from $p18^{-/-}$ mice (16 weeks of age) was approximately 40% higher than the number from wild-type littermate mice. Although most of the islets of $p18^{-/-}$ mice (72.1%) were small, there were significantly more
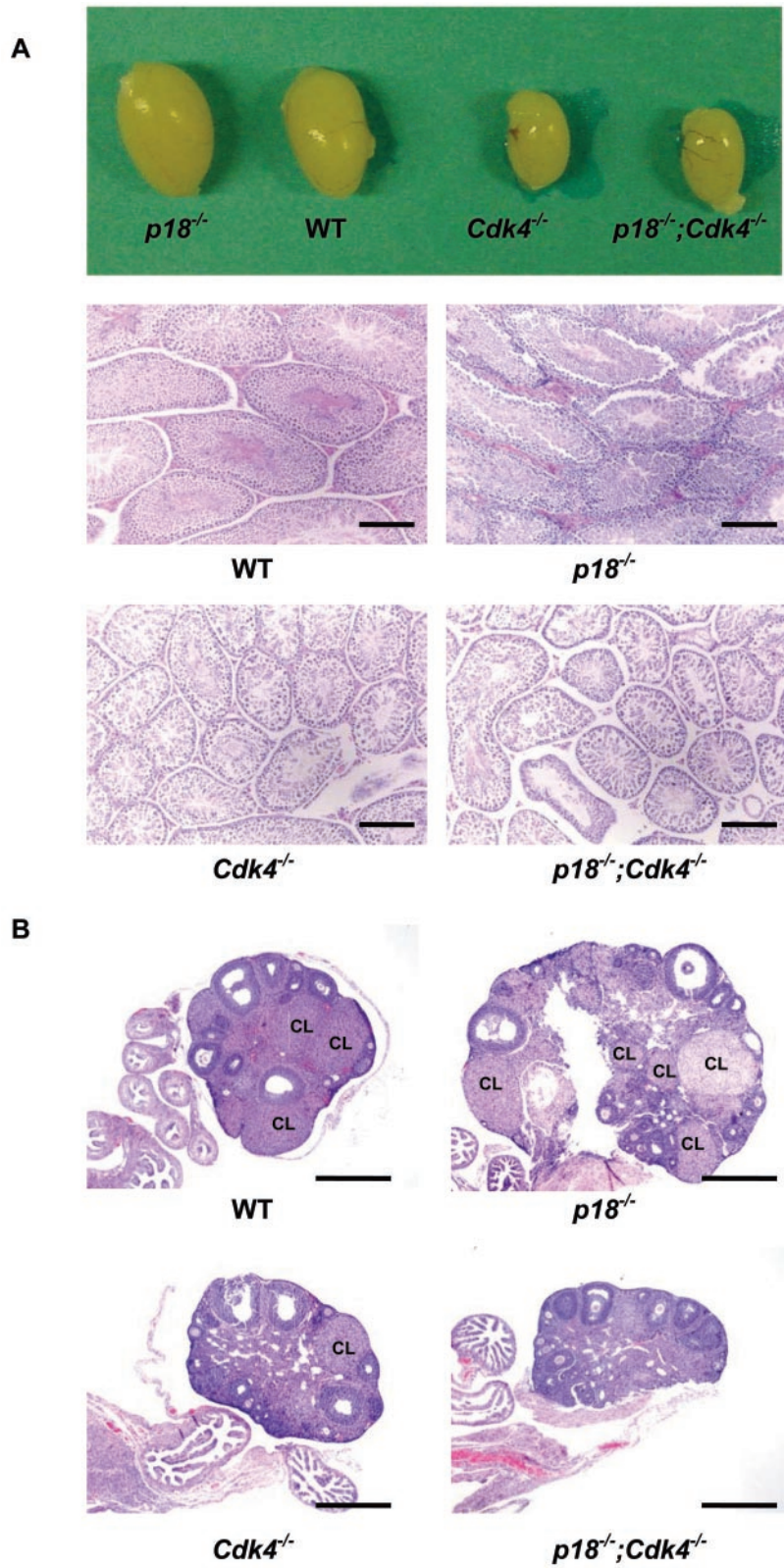
FIG. 2. Infertility of $p18^{-/-}$; $Cdk4^{-/-}$ mice. (A) Gross appearance of testes from 5-month-old mice (upper row) and sections of testes stained with hematoxylin and eosin (HE) from mice of different genotypes at 5 months of age. WT, wild type. Bars, 200 μm. (B) Sections of ovary stained with HE from mice of different genotypes from the same litter at 4 months of age. The CL is indicated. Bars, 1 mm.

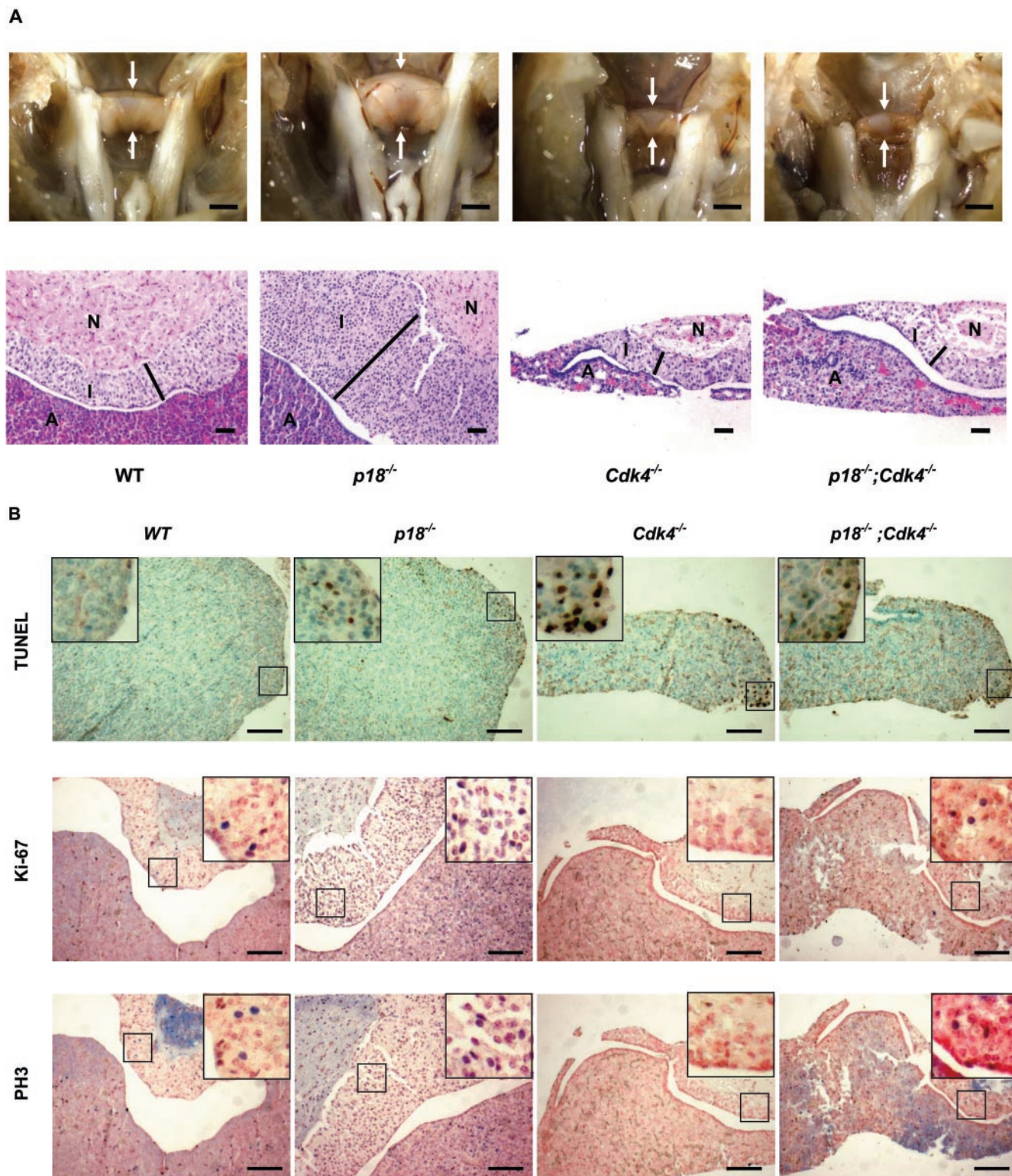A

WT
p18⁻/⁻
Cdk4⁻/⁻
p18⁻/⁻;Cdk4⁻/⁻

B

FIG. 3. Abnormalities of the pituitary in *p18* and *Cdk4* mutant mice. (A) Pituitary glands (arrows) from mice of different genotypes from the same litter were microscopically examined at 4 months of age either directly (top row) or after hematoxylin and eosin staining (bottom row). The anterior lobe (A), intermediate lobe (I), and neurohypophysis (N) are indicated. The width of the intermediate lobe is shown by the black bars. WT, wild type. Bars, 1 mm (top row) and 50 μm (bottom row) (B) Series of sections of pituitary glands from littermate mice of different genotypes at 4 months of age were examined for apoptotic cell death by TUNEL assay (top), for cell proliferation by Ki-67 staining (middle), and for mitotic activity by immunostaining with an antibody recognizing phosphorylated histone H3 (bottom). Arrows, positive (brown staining or blue staining) cells. Bars, 200 μm.

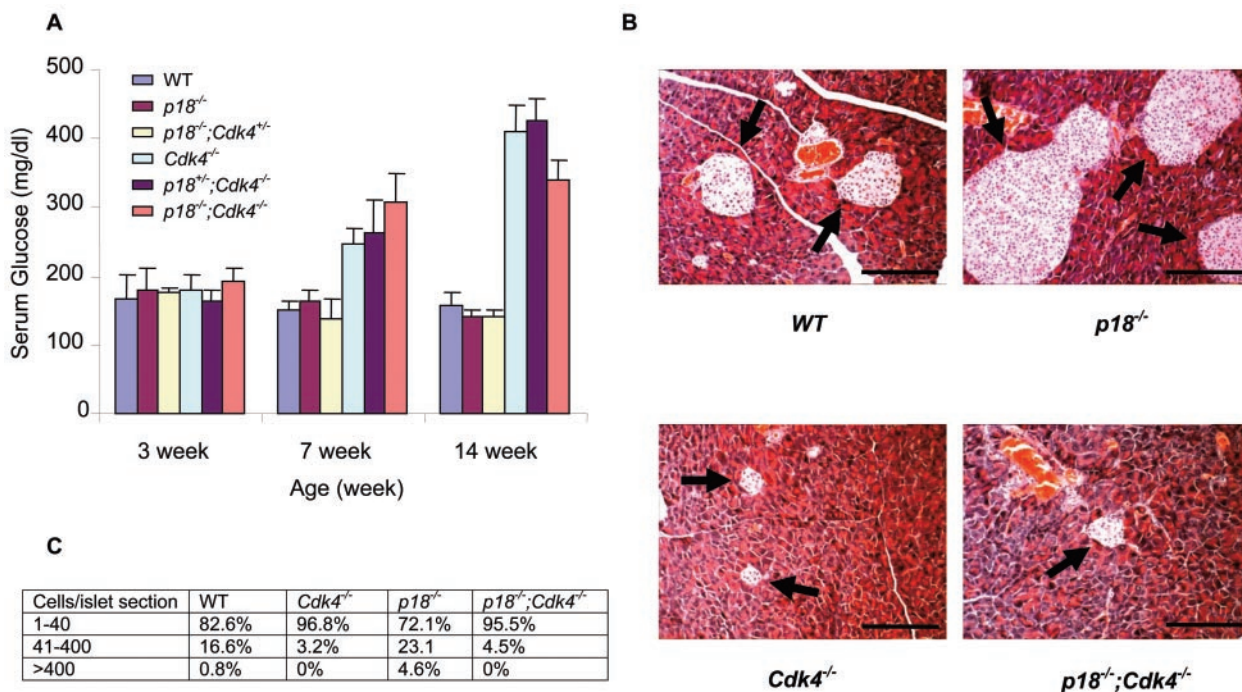| Cells/islet section	WT	$Cdk4^{-/-}$	$p18^{-/-}$	$p18^{-/-};Cdk4^{-/-}$
1-40	82.6%	96.8%	72.1%	95.5%
41-400	16.6%	3.2%	23.1	4.5%
>400	0.8%	0%	4.6%	0%

FIG. 4. Loss of *p18* did not rescue diabetes of *Cdk4*-deficient mice. (A) Glucose levels in serum of 3-, 7-, and 14-month-old mice. Three or four mice from each genotype were examined. Standard deviation bars are indicated. (B) hematoxylin and eosin staining of pancreatic sections obtained from mice of different genotypes from the same litter at 4 months age of. Arrows, islets. WT, wild type. (C) Percentages of islets containing 1 to 40, 41 to 400, or more than 400 cells/section in mice of different genotypes at 4 months of age. Two hundred fifty islets from WT and *p18*$^{-/-}$ pancreases and 100 islets from *Cdk4*$^{-/-}$ and *p18*$^{-/-}$; *Cdk4*$^{-/-}$ pancreases were counted. Bars, 200 μm.

medium-sized (23.1%) and especially large-sized islets (4.6%) than were found in wild-type mice (Fig. 4B). Pancreases of *p18*$^{-/-}$; *Cdk4*$^{-/-}$ mice are very similar to those of *Cdk4*$^{-/-}$ mice; islets were sparsely located in the *p18*$^{-/-}$; *Cdk4*$^{-/-}$ pancreas, and the number of islets was comparable to the number in *Cdk4*$^{-/-}$ mice. For the *p18*$^{-/-}$; *Cdk4*$^{-/-}$ islets, 95.5% were small and only 4.5% could grow to medium size. No large-sized islets were found in *p18*$^{-/-}$; *Cdk4*$^{-/-}$ pancreases (Fig. 4C). Consistent with the defects in pancreatic development and function, *p18*$^{-/-}$; *Cdk4*$^{-/-}$ mice also developed diabetes from about 7 weeks of age (Fig. 4A). Loss of *p18* did not rescue defective pancreatic islet (β-cell) development. Instead, loss of *Cdk4* abolished all hyperplastic growth of pancreatic islet β cells caused by *p18* loss.

Cdk4 and p18 are dispensable for retinal development. Rb family proteins play an important role in retinal development. Inactivation of *Rb* and/or *p107* leads to multiple retinal dysplasia (31, 32, 41, 50). Loss of *cyclin D1* results in small eyes with thin retinas and a dramatic reduction in cell numbers in all layers of the retina as the result of decreased cell proliferation and increased apoptosis (13, 30, 46), implying a possible role for CDK4 and/or CDK6 and INK4 in normal retinal development through controlling the phosphorylation and activity of Rb proteins. Close examination of retinas of *Cdk4*$^{-/-}$, *p18*$^{-/-}$, and *p18*$^{-/-}$; *Cdk4*$^{-/-}$ mice did not identify any obvious abnormality (Fig. 5), indicating that CDK4 and p18 are both dispensable in retinal development. These findings suggest that CDK6 is likely to play a prominent role in controlling retinal development. In contrast to *cyclin D1*-deficient retinas, *p27*-

deficient retinas developed hyperproliferation and displayed disorganized cellular layers and protrusions of the outer photoreceptor cell layer into the rod-and-cone layer (10, 48). Simultaneous deletion of *p27* and *cyclin D1* rescued hyperproliferation and hypocellularity caused by the deletion of either gene and largely restored normal retinal development (48), providing further evidence for a functional distinction between p18 and p27 in controlling cyclin D-dependent kinases.

Cdk4 is required for development of kidney, adrenal glands, and seminal vesicles, and deletion of p18 does not restore the developmental defects of these organs in Cdk4 null mice. Deletion of *Cdk4* resulted in small kidney, adrenal glands, and seminal vesicles. These three organs are 55% of the sizes of the wild-type counterparts. Histologically, loss of *Cdk4* resulted in severe hypoplasia in these organs (Fig. 6 and data not shown). In contrast, deletion of the *p18* gene resulted in increased sizes of kidney, adrenal glands, and seminal vesicles (15) (Fig. 6). Deletion of *p18* did not rescue hypoproliferative phenotypes associated with *Cdk4*-deficient mice. The kidneys, adrenal glands, and seminal vesicles of *p18*$^{-/-}$; *Cdk4*$^{-/-}$ mice are smaller than those of both wild-type and *p18*$^{-/-}$ mice and are similar to those of *Cdk4*$^{-/-}$ mice (Fig. 6).

Loss of p18 rescues S phase delay in MEFs lacking Cdk4. Loss of CDK4 leads to a delay of the initiation of DNA replication of serum-deprived MEFs (49), indicating a role for CDK4 in mediating the exit of MEFs from quiescence. The studies on the signaling pathways regulating CDK4 and CDK6 have been mostly focused on the activation of cyclin D gene expression (34). Whether any INK4 protein plays a role in this
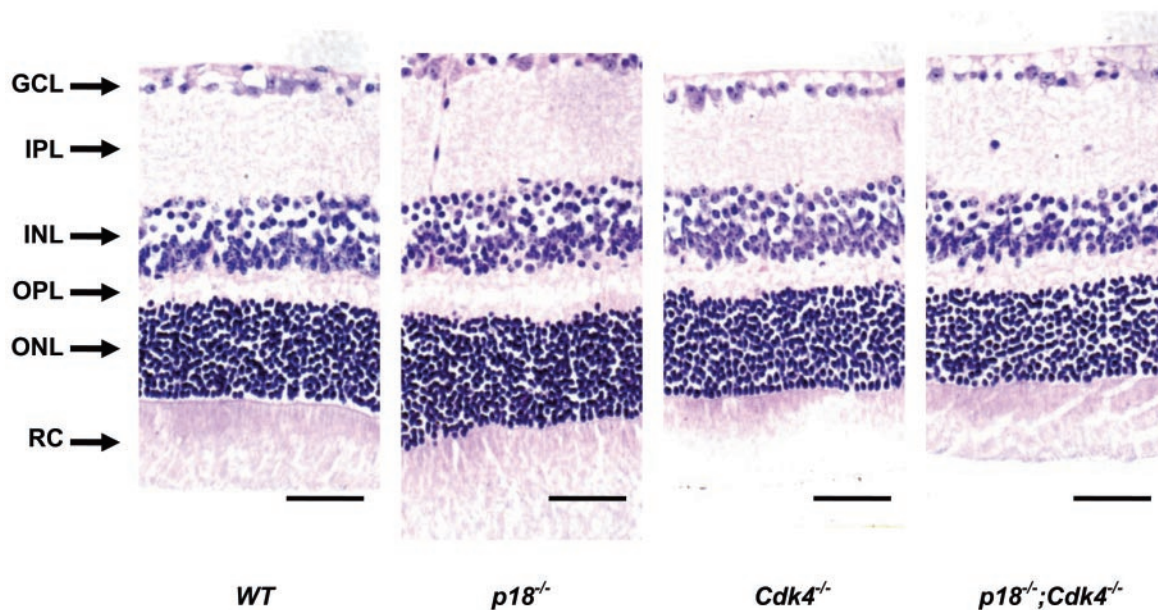
FIG. 5. *p18* and *Cdk4* are functional dispensable for normal development of the retina. Shown are histologic sections of retinas derived from mice of different genotypes at 5 months of age. The ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and rod-and-cone layer (RC) are indicated. Bars, 50 μm.

regulation has not been reported. To determine the function of p18 in the G_0/G_1-to-S transition, we derived MEFs from p18-deficient mice and examined their response to serum starvation by flow cytometry. Compared to what was found for wild-type MEFs, deletion of p18 resulted in an accelerated progression through the G_1 phase and entry into S phase. While 19.7 and 26.7% of $p18^{-/-}$ MEFs have started DNA replication at 18 and 21 h post-serum stimulation, respectively, only 17.3 and 21.8% of wild-type MEFs were BrdU positive (Fig. 7). Twenty-four hours post-serum stimulation, while more (24.2%) wild-type MEFs continued to enter into S phase, there was a decrease of BrdU-positive $p18^{-/-}$ MEFs, indicating that most of them had completed DNA replication. The cell cycle kinetics of $p18^{-/-}$ MEFs in response to serum deprivation-stimulation is in contrast to that of $Cdk4^{-/-}$ MEFs, which, as previously reported, displayed a delayed entry into S phase relative to wild-type MEFs that was similar to that of $p27^{-/-}$ MEFs, which also had an accelerated progression through the G_1 phase.

The function of *p18* in regulating the cellular response to serum starvation-stimulation has not been previously reported. To confirm this finding, we determined the expression of *p18* gene transcripts during G_1 progression. Early-passage (younger than passage 4) MEFs were serum starved for 72 h and then stimulated by the addition of 10% FBS. We previously reported that the *p18* gene expresses two species of message though promoter switching, a translation-competent short transcript and a translation-attenuated long transcript, due to the presence of a 1.2-kb 5′-untranslated region encoded by exon 1 (39). In serum-starved quiescent cells, the short transcript accumulates to a low level while the long 2.4-kb transcript was barely detectable (Fig. 7C). Following serum stimulation, the levels of both transcripts increased and

reached a peak around the G_1/S boundary. This result suggests that the expression of p18 is regulated, at least in part, by a transcriptional control and provides a molecular basis for the accelerated G_1-to-S progression of $p18^{-/-}$ MEFs.

To determine the functional interaction between CDK4 and its two regulators, p18 and p27, we derived $p18^{-/-}$; $Cdk4^{-/-}$ and $p27^{-/-}$; $Cdk4^{-/-}$ double-mutant MEFs and determined their cell cycle kinetics for serum deprivation and stimulation. Consistent with a previous report (49), the $p27^{-/-}$; $Cdk4^{-/-}$ MEFs displayed cell cycle kinetics intermediate between those of $Cdk4^{-/-}$ and $p27^{-/-}$ MEFs, indicating that deletion of p27 partially restored the delayed S-phase entry caused by $Cdk4$ loss (Fig. 7A and B). Surprisingly, $p18^{-/-}$; $Cdk4^{-/-}$ MEFs exhibited kinetics more similar to that of $p18^{-/-}$ MEFs: 20.1 and 25.4% of cells were stained positive for BrdU at 18 and 21 h post-serum stimulation. These results are consistent with the idea that *p18* plays a critical role in controlling the serum response in MEFs by regulating not only CDK4 but also another target, likely CDK6, and that delay in S phase entry due to loss of *Cdk4* could be compensated by an increase of CDK6 activity.

DISCUSSION

INK4 proteins specifically bind to and negatively regulate the activity of CDK4 and CDK6 (44), and cyclin Ds-CDK4/6 complexes phosphorylate and functionally inactivate Rb family proteins (9, 12, 22). Ectopic overexpression of individual *INK4* genes causes a G_1 cell cycle arrest with a correlative dependency on the intact Rb (16), and loss of either *Rb* or a combination of *p107* and *p130* effectively canceled the G_1 arrest caused by INK4 overexpression (3, 24, 35). Together, these findings led to the widely accepted notion that, at least in
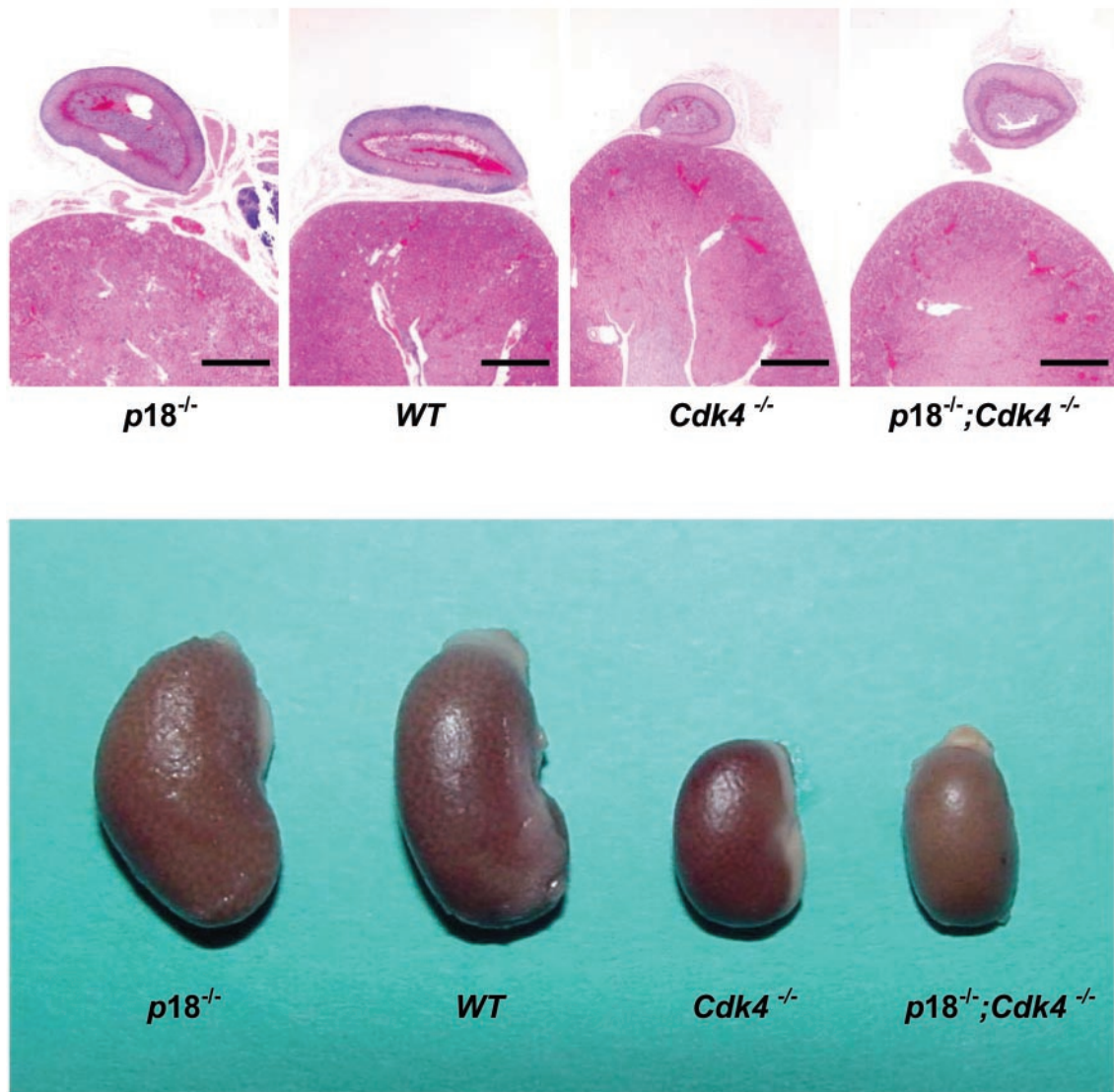
FIG. 6. Histological analysis of kidneys and adrenal glands of *p18* and *Cdk4* mutant mice. (Top) Histologic sections of kidneys and adrenal glands derived from mice of different genotypes at 4 months of age were examined microscopically. (Bottom) Gross appearance of kidneys and adrenal glands. WT, wild type. Bars, 1 mm.

cultured cells and assays for controlling G_1-to-S transition, the primary functional targets of INK4 are CDK4 and CDK6 and the primary functional targets of CDK4 and CDK6 are the pocket proteins. Hence, there may exist in vivo a linear INK4-CDK4/6-Rb G_1 control pathway in mammalian cells. The notion that CDK4 and CDK6 are the primary targets of INK4 function, however, has not been tested experimentally in any in vivo setting, due largely to the lack of cells lacking both CDK4 and CDK6. Two additional proteins, orphan steroid receptor Nur77 (5) and the p65^{RelA} subunit of NF-κB (52), were reported to bind with p19^{Ink4d} and p16^{Ink4a}, respectively, although the functional consequence and physiologic significance of both interactions remain unclear. Generation and characterization of *p18*- and *Cdk4*-deficient mutant mouse strains and development of a series of consistent phenotypes in these two strains of mice provide an opportunity to determine the functional dependency of INK4 on CDK4 in vivo. In this paper, we provide the first genetic evidence that the developmental function of an INK4 protein is dependent on CDK4.

We have examined in *p18$^{-/-}$*, *Cdk4$^{-/-}$*, and *p18$^{-/-}$*; *Cdk4$^{-/-}$* mice body weight and six cell types, tissues, or organs which developed highly penetrant phenotypes because of the loss of *p18* alone: testis, ovary, pituitary, endocrine pancreas, kidney, and adrenal gland. In all six cases, simultaneous loss of *Cdk4* virtually canceled all *p18* loss-induced defects. These results provide direct evidence that the function of p18^{Ink4c} is dependent on CDK4 in vivo. Many of these hyperproliferative defects associated with *p18* loss were also developed in *p27*-deficient mice to a similar extent with a nearly complete penetrance, including body weight increase; pituitary hyperplasia and tumor development; and enlargement of thymus, spleen, testis, and ovary (14, 23, 37). Notably, the body weight in-
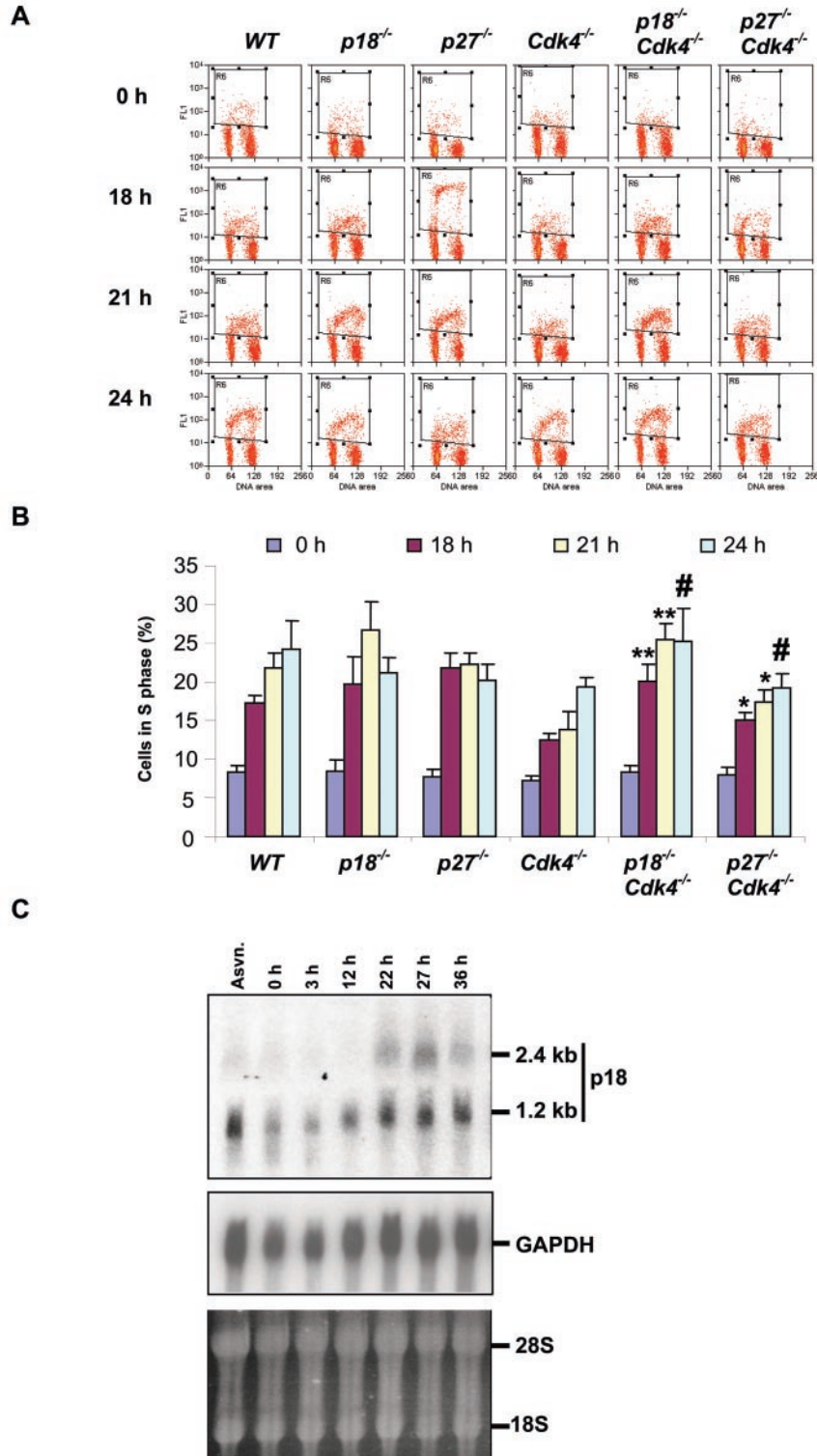
FIG. 7. *p18* has a function in serum response in MEFs that is not dependent on *Cdk4*. (A) Cell cycle analysis by staining with BrdU and propidium iodide (PI). MEFs were starved for 72 h (time zero), followed by stimulation of cells with medium containing 10% serum. Cells were pulse-labeled and harvested at the times indicated after stimulation. Cells were stained with an anti-BrdU antibody and PI and subjected to flow cytometry. BrdU-positive cells are boxed. WT, wild type. (B) The percentage of cells in S phase from each time point was plotted on the basis of flow-cytometric analysis. Results are average values of three independent experiments. Error bars indicate standard deviations of the means. ∗∗, $P < 0.01$, for comparison of $p18^{-/-}$; $Cdk4^{-/-}$ MEFs to $Cdk4^{-/-}$ MEFs at the same times after serum restimulation; ∗, $P < 0.05$, for comparison of $p27^{-/-}$; $Cdk4^{-/-}$ MEFs to $Cdk4^{-/-}$ MEFs at the same times after serum restimulation; #, $P > 0.05$, for comparison of $p18^{-/-}$; $Cdk4^{-/-}$ or $p27^{-/-}$; $Cdk4^{-/-}$ MEFs to $Cdk4^{-/-}$ MEFs at the same time point (Student's t test). (C) Induction of *p18* gene expression during G$_1$ progression. Total RNA was isolated from asynchronously growing (Asyn.) and serum-starved and stimulated MEFs. Twenty micrograms of each RNA sample was resolved on a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized with a probe derived from mouse *p18* coding region.

crease, or the gigantism phenotype, caused by *p27* loss was only partially canceled in *p27*$^{-/-}$; *Cdk4*$^{-/-}$ double-mutant mice (Fig. 1). This result is consistent with and provides genetic support for the model that, while INK4 regulates only CDK4 and CDK6, CIP/KIP proteins have an additional target, CDK2. The role of p27 (and p21) in the regulation of *Cdk4* is currently controversial (38), and it has been suggested that p27 has opposing roles as an activator (6) and as an inhibitor (1). Our results that the body weights of *p27*$^{-/-}$; *Cdk4*$^{-/-}$ double-mutant mice and the cell cycle kinetics of *p27*$^{-/-}$; *Cdk4*$^{-/-}$ MEFs exhibited a phenotype intermediate between that of either single mutant are in agreement with an oppose function of these two genes and support the notion that p27 is an inhibitor of Cdk4. In addition to tumor suppression, the Rb pathway controls cell differentiation, tissue development, and organ size. Of various *p18* loss-caused defects that were canceled by the simultaneous loss of *Cdk4*, the body weight increase, pancreatic islet number increase and size enlargement, and ovary enlargement are defects in animal development and organ size regulation, not tumor growth, indicating that the functional dependency of *p18* on *Cdk4* is not restricted to tumor suppression and may extend into other cellular processes.

The only defect caused by *p18* loss that was not significantly alleviated by the loss of *Cdk4* is the accelerated G$_1$-to-S progression of MEFs following serum deprivation and stimulation: *p18*$^{-/-}$; *Cdk4*$^{-/-}$ MEFs displayed cell cycle kinetics more similar to that of *p18*$^{-/-}$ MEFs (Fig. 7). In the same assay, the *p27*$^{-/-}$; *Cdk4*$^{-/-}$ MEFs exhibited intermediate cell cycle kinetics, indicating, again, that deletion of *Cdk4* partially reduced accelerated G$_1$-to-S progression caused by *p27* loss. The most likely explanation for the lack of significant rescue of *p18* loss-caused defects by *Cdk4* loss is that in MEFs, unlike other adult tissues and differentiated cells, *Cdk6* may play a significant role in regulating G$_1$ progression following serum starvation-stimulation, either in its own right or when induced in a compensatory manner after *Cdk4* loss, therefore sensitizing the MEFs to *p18* loss. Consistent with this idea, CDK6 is readily detectable in MEFs and CDK6-p27 complexes were noticeably increased in *Cdk4* null MEFs (49), suggesting a compensatory regulation of p27, and thus Cdk2, by CDK6 in the absence of CDK4. The in vivo function of CDK6 in the whole animal and how widely CDK6 compensates for CDK4 loss in other cell types and tissues remain unclear. Our results that *Cdk4* loss canceled all six defects caused by *p18* loss that we have examined suggest that *Cdk6* plays a relatively minor role in vivo in mediating the function of *p18*.

Wild-type MEFs accumulate in either G$_1$ phase of the cell cycle or a quiescence state in response to serum deprivation and then reenter the cell cycle and travel through the G$_1$ phase with consistent kinetics: DNA replication starts approximately 14 to 16 h after serum stimulation. Loss of *p18* accelerated G$_1$ progression and initiation of DNA replication (Fig. 7), revealing a previously unrecognized function of *p18* in regulation of the serum response in MEFs. Supporting this function of *p18* is the observation that *p18* mRNA is induced during reentry of serum-deprived MEFs into the cell cycle (Fig. 7). The mechanism responsible for p18 induction during G$_1$ progression remains to be determined but does not appear to be dependent on the function of E2F1. Notably, similar percentages of *p18*$^{-/-}$ and wild-type MEFs (7 to 8%) were stained positive for

BrdU after 72 h of serum starvation (Fig. 7), indicating that *p18*$^{-/-}$ MEFs were arrested in the G$_0$ quiescent state as efficiently as wild-type MEFs. Likewise, loss of *p27*, which also accelerated G$_1$ progression following serum stimulation, did not impair the ability of MEFs to enter and maintain a stable quiescence upon serum deprivation. Differently, in response to serum deprivation, the *Rb*$^{-/-}$; *p107*$^{-/-}$; *p130*$^{-/-}$ triple-deficient MEFs continue to incorporate BrdU and then undergo apoptotic cell death (7, 43), indicating an essential function of pocket proteins, and by extension implying a function of a CDK inhibitor(s), in causing and/or maintaining G$_1$ arrest under growth-inhibiting conditions. Lack of a significant defect in entering into and maintaining G$_1$ arrest upon serum starvation in p18-deficient MEFs is consistent with its decreased low-level expression in quiescent cells (Fig. 7). It remains to be determined whether, during the cellular response to growth factor deprivation-stimulation, p18 and p27 alone are sufficient to cause and maintain a G$_1$ arrest, but both are required for a proper reentry of quiescent cells into the proliferation cycle. Alternatively, our results suggest that another CDK inhibitor protein separately controls the entry into and maintenance of G$_1$ arrest while p18 and p27 regulate the reentry into the cell cycle.

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