

# Wide spectrum of tumors in knock-in mice carrying a Cdk4 protein insensitive to INK4 inhibitors

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**We have introduced a point mutation in the first coding exon of the locus encoding the cyclin-dependent kinase 4 (Cdk4) by homologous recombination in embryonic stem cells. This mutation (replacement of Arg24 by Cys) was first found in patients with hereditary melanoma and renders Cdk4 insensitive to INK4 inhibitors. Here, we report that primary embryonic fibroblasts expressing the mutant Cdk4<sup>R24C</sup> kinase are immortal and susceptible to transformation by Ras oncogenes. Moreover, homozygous Cdk4<sup>R24C/R24C</sup> mutant mice develop multiple tumors with almost complete penetrance. The most common neoplasia (endocrine tumors and hemangiosarcomas) are similar to those found in pRb<sup>+/-</sup> and p53<sup>-/-</sup> mice. This Cdk4 mutation cooperates with p53 and p27<sup>Kip1</sup> deficiencies in decreasing tumor latency and favoring development of specific tumor types. These results provide experimental evidence for a central role of Cdk4 regulation in cancer and provide a valuable model for testing the potential anti-tumor effect of Cdk4 inhibitors *in vivo*.**

**Keywords:** animal models/cell cycle regulation/cyclin-dependent kinase/INK4 inhibitors/tumor development

## Introduction

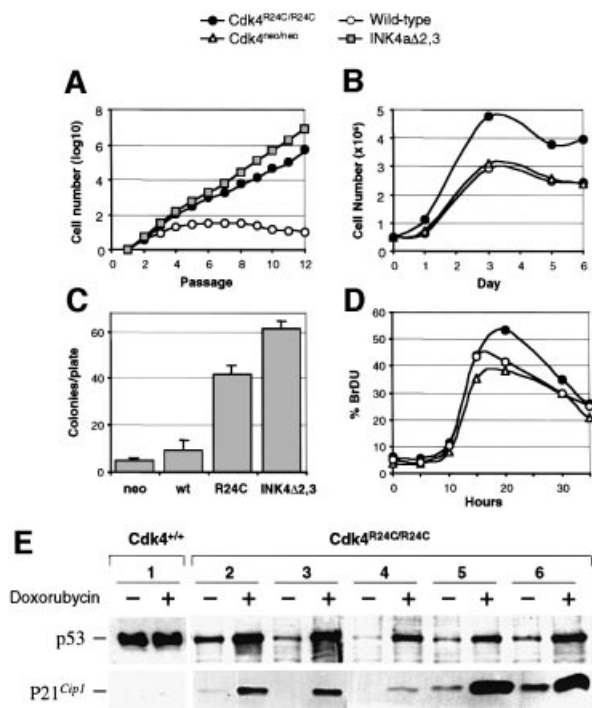
The central role of cyclin/cyclin-dependent kinase (Cdk) complexes and their regulation in the modulation of cell cycle progression has been widely recognized (Morgan, 1995; Sherr, 2000). Cdk4 and Cdk6 are two cell cycle kinases involved in cell cycle initiation of resting cells as a response to mitogenic signals, as well as in the progression through the G<sub>1</sub>/S transition. These kinases associate with the growth factor-regulated D-type cyclins (D1, D2, D3) to form active complexes that phosphorylate and inactivate the retinoblastoma protein (pRb). The molecular mechanisms involved in Cdk regulation have been extensively studied (Morgan, 1995; Pavletich, 1999; Sherr and Roberts, 1999). For instance, Cdks can be regulated by activating and inactivating phosphorylations, binding of inhibitory proteins and interaction with chaperone

molecules that provide a proper folding and transport to the nucleus.

Two families of Cdk inhibitors have been described based on functional and structural homologies. The INK4 family of inhibitors, p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup> and p19<sup>INK4d</sup>, are structurally characterized by ankyrin repeats, and specifically bind to Cdk4 and Cdk6 preventing their interaction with D-cyclins. The second group of inhibitors, known as the Cip/Kip family, is composed of three members, p21<sup>Cip1/Waf1/Sdi1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>. These proteins are primary inhibitors of Cdk2 activity by forming ternary complexes with cyclin E- and cyclin A-bound Cdk2 proteins. Interestingly, members of the Cip/Kip family can also bind and form ternary complexes with cyclin D-Cdk4/6 kinases. However, in this case the ternary complexes can retain kinase activity. These and other observations have led to the hypothesis that cyclin D-Cdk4/6 complexes may participate in the sequential activation of downstream cyclin E-Cdk2 complexes by removing circulating Cip/Kip molecules that may bind to cyclin E-Cdk2 complexes (reviewed by Sherr and Roberts, 1999).

Increasing evidence, obtained primarily from human tumors, indicates that regulators of the cell cycle including cyclins, Cdks and Cdk inhibitors are either themselves targets for genetic modification in cancer or are disrupted by other oncogenic events (Hirama and Koeffler, 1995; Sherr, 2000). Most of these mutations result in deregulation of the G<sub>1</sub> kinases, Cdk4 and Cdk6 (Sherr, 2000). For instance, overexpression of cyclins or deletion of the *INK4a-ARF* locus are among the most frequent alterations in human malignancies (Peters, 1994; Ruas and Peters, 1998). Deletion of this locus results in loss of two different proteins, p16<sup>INK4a</sup> and p19<sup>ARF</sup>. p19<sup>ARF</sup> is a tumor suppressor involved in stabilization of p53 (Sharpless and DePinho, 1999; Sherr, 2000). The adjacent *INK4b* locus, which encodes p15<sup>INK4b</sup>, is also frequently co-deleted as well as inactivated by promoter hypermethylation (Ruas and Peters, 1998). Expression of Cdk4 or Cdk6 is also altered in human cancer by mechanisms involving gene amplification or chromosomal translocations (Khatib *et al.*, 1993; Schmidt *et al.*, 1994; Chilos *et al.*, 1998; Corcoran *et al.*, 1999). Finally, miscoding mutations in the *CDK4* locus have been observed in familial and sporadic melanoma (Wolfel *et al.*, 1995; Zuo *et al.*, 1996). These mutations render a Cdk4 protein whose kinase activity becomes insensitive to INK4 inhibitors.

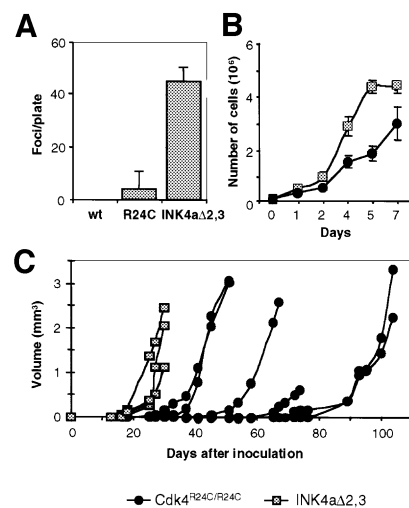
Recently, the role of some of these proteins in normal homeostasis as well as in tumor development has been studied in gene-targeted mice. Strains lacking each of the INK4 inhibitory proteins have already been described (for a review see Malumbres *et al.*, 2000a). Whereas p16<sup>INK4a</sup> and p15<sup>INK4b</sup> are rather weak tumor suppressors, p18<sup>INK4c</sup>-deficient mice are highly susceptible to pituitary as well as



**Fig. 1.** Growth properties of Cdk4<sup>R24C/R24C</sup> MEFs. MEFs derived from wild-type (open circles), Cdk4<sup>R24C/R24C</sup> (filled circles), Cdk4<sup>neo/neo</sup> (open triangles) and INK4a $\Delta$ 2,3 (gray squares) mice were utilized. (A) Growth of MEFs according to a 3T3 protocol; (B) cell density; (C) plating efficiency; (D) DNA synthesis after serum re-stimulation; and (E) functional analysis of the p53 pathway in immortal clones derived from wild-type MEFs (clone 1) or Cdk4<sup>R24C/R24C</sup> MEFs (clones 2–6). Immortal wild-type MEFs show overexpression of a p53 protein that is not able to induce p21<sup>Cip1</sup> after treatment with doxorubicin. All five Cdk4<sup>R24C/R24C</sup> clones display normal p53 and p21<sup>Cip1</sup> induction after doxorubicin treatment. Results shown in (A–D) correspond to an average of three to five different experiments.

to other types of tumors (Franklin *et al.*, 1998; Latres *et al.*, 2000; Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001). No tumors have been detected in p19<sup>INK4d</sup> knock-out mice (Zindy *et al.*, 2000). The function of D1 and D2 cyclins has also been investigated. Mice lacking D1 cyclin have reduced size and display severe retinopathy due to impaired development of the retina and limited development of mammary acinar cells during pregnancy (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). On the other hand, D2 cyclin deficiency results in female sterility due to a defect in granulosa cell proliferation and male hypoplastic testis (Sicinski *et al.*, 1996). Finally, the role of Cdk4 *in vivo* has only been investigated in the case of Cdk4 (Rane *et al.*, 1999; Tsutsui *et al.*, 1999). Mice lacking Cdk4 are viable but have reduced size and severe proliferative defects in certain endocrine cells, primarily testicular Leydig cells and pancreatic  $\beta$ -cells (Rane *et al.*, 1999; J.Martin, S.L.Hunt, P.Dubus, R.Sotillo, M.Malumbres, S.Ortega and M.Barbacid, in preparation).

To understand the role of Cdk4 in neoplastic development, we have engineered a single miscoding mutation in the first exon of the mouse Cdk4 locus by gene targeting (Rane *et al.*, 1999). The resulting knock-in mice, Cdk4<sup>R24C/R24C</sup>, express an endogenous Cdk4 protein that carries the Arg to Cys substitution (Cdk4R24C), the same mutation found in melanoma patients (Wolfel *et al.*, 1995;



**Fig. 2.** Susceptibility of primary MEFs to transformation by Ras oncogenes. (A) Number of transformed foci per plate in wild-type (wt), Cdk4<sup>R24C/R24C</sup> or INK4a $\Delta$ 2,3 MEFs after transfection with oncogenic H-Ras. (B) Growth curves of Ras-transformed clones derived from Cdk4<sup>R24C/R24C</sup> MEFs (filled circles) and INK4a $\Delta$ 2,3 MEFs (squares). Data represent the average and standard deviation of two experiments with two different clones. (C) Growth of tumors after subcutaneous injection of individual Ras-transformed clones in nude mice. No tumors were observed after injection of non-transfected Cdk4<sup>R24C/R24C</sup> MEFs.

Zuo *et al.*, 1996). Here, we report that primary embryonic fibroblasts expressing the endogenous Cdk4R24C allele are immortal and susceptible to oncogenic transformation. In addition, homozygous, as well as heterozygous, Cdk4R24C mice develop a wide spectrum of tumors from different cell lineages. This mutation cooperates with deficiencies in other tumor suppressor genes such as p53 or p27<sup>Kip1</sup>. These results highlight the central role of Cdk4 regulation in cell transformation and provide a valuable model for developing cancer therapy strategies based on Cdk4 inhibition.

## Results

### Growth properties of Cdk4<sup>R24C/R24C</sup> mouse embryo fibroblasts

Mouse embryonic fibroblasts (MEFs) obtained from Cdk4<sup>R24C/R24C</sup> mice express normal levels of a Cdk4 protein that does not bind p16<sup>INK4a</sup> and induces hyperphosphorylation of pRb (Rane *et al.*, 1999). When maintained in a 3T3 passage scheme, these MEFs grow faster than wild-type MEFs (Figure 1). The population doubling time in the exponential phase of proliferation is ~18–20 h for Cdk4<sup>R24C/R24C</sup> cells, 16–18 h for control INK4a–ARF-deficient cells (MEFs derived from INK4a $\Delta$ 2,3 mice that lack p16<sup>INK4a</sup> and p19<sup>ARF</sup> proteins; Serrano *et al.*, 1996) and 28–30 h for wild-type cells. Cdk4<sup>R24C/R24C</sup> MEFs show an almost undetectable cell period, and do not enter into senescence after many passages (Figure 1A), a result reminiscent of that observed with INK4a–ARF mutant cells (Serrano *et al.*, 1996). Cdk4<sup>R24C/R24C</sup> cells display other properties characteristic of a higher proliferative potential. Cdk4<sup>R24C/R24C</sup> monolayers achieve higher cellular densities (Figure 1B), show a higher efficiency in colony formation when seeded at low density (Figure 1C), and a higher number of cells enter

**Table I.** Tumor incidence in Cdk4<sup>R24C/R24C</sup> mice

Tumor type	Spontaneous death ( <i>n</i> = 105)		Killed at 14–16 months ( <i>n</i> = 50)	
Mesenchymatous tumors <sup>a</sup>	71	(67%)	16	(32%)
hemangiosarcomas	59	(56%)	11	(22%)
other sarcomas <sup>b</sup>	6	(6%)	2	(4%)
benign mesenchymatous tumors <sup>c</sup>	8	(8%)	3	(6%)
Epithelial endocrine tumors <sup>a</sup>	58	(55%)	34	(68%)
Leydig cell tumors (males only <sup>d</sup> )	38	(62%)	21	(70%)
pancreatic endocrine tumors	32	(31%)	21	(42%)
pituitary tumors	23	(22%)	3	(6%)
other endocrine tumors <sup>e</sup>	2	(2%)	0	(0%)
Epithelial non-endocrine tumors <sup>a</sup>	25	(24%)	21	(42%)
lung adenomas/adenocarcinomas	19	(18%)	16	(32%)
hepatocellular tumors <sup>f</sup>	6	(6%)	6	(12%)
other epithelial tumors <sup>g</sup>	3	(3%)	5	(10%)
Lymphoid/myeloproliferative tumors	3	(3%)	1	(2%)
lymphoma	2	(2%)	0	(0%)
mast cell	1	(1%)	1	(2%)

Numbers in parentheses indicate tumor incidence.

<sup>a</sup>The total number of mice with epithelial endocrine and non-endocrine tumors as well as mice with mesenchymatous tumors is less than the sum of mice with each individual tumor type due to the presence of multiple tumors in some of the animals.

<sup>b</sup>Fibrosarcoma, liposarcoma, osteosarcoma, malignant fibrous histiocytoma and undifferentiated sarcoma.

<sup>c</sup>Lipoma, osteoma, etc.

<sup>d</sup>Number of male mice was *n* = 61 and *n* = 30, respectively.

<sup>e</sup>Thyroid and granulosa cell tumors.

<sup>f</sup>All hepatocellular tumors developed in males (10–20% incidence in males).

<sup>g</sup>Gut, exocrine glands and kidney tumors.

simultaneously into S phase after starvation and re-stimulation with serum (Figure 1D).

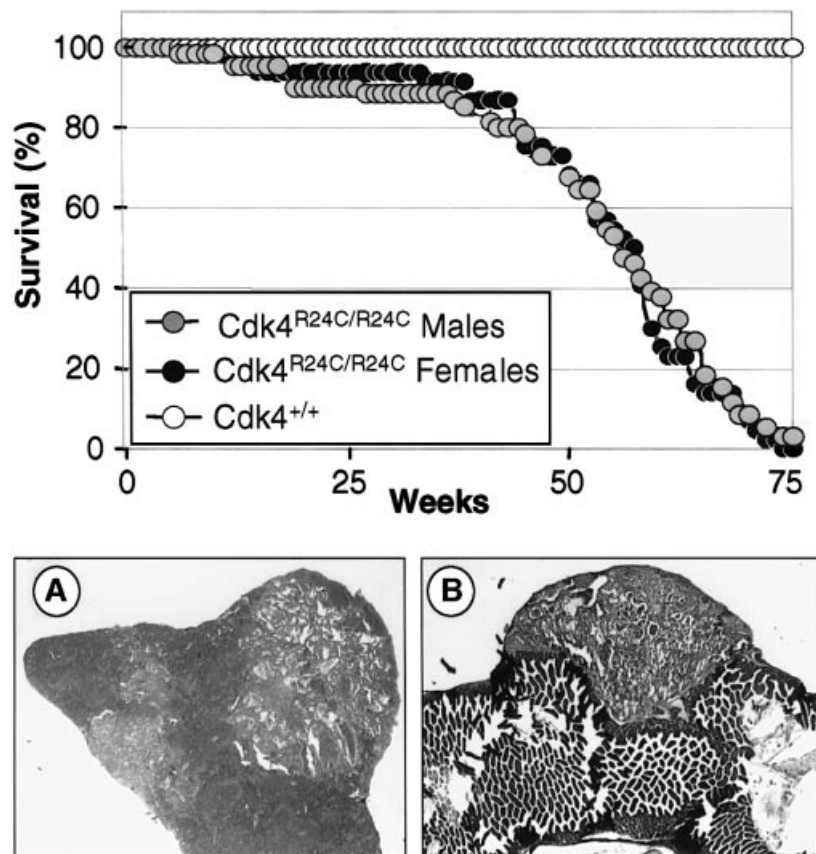
It has been proposed that replicative senescence in INK4a-ARF<sup>-/-</sup> MEFs results from the p53-dependent growth inhibitory effect of p19<sup>ARF</sup> (Kamijo *et al.*, 1997; Sherr and DePinho, 2000), since spontaneous immortalization of MEFs is usually accompanied by either mutation of p53 or deletion of the *INK4a-ARF* locus. To examine the functional status of the p53 pathway, we have analyzed the p53-dependent response to DNA damage induced after doxorubicin insult in Cdk4<sup>R24C/R24C</sup> MEFs. As illustrated in Figure 1E, each (5/5) of the immortal Cdk4<sup>R24C/R24C</sup> MEF clones examined displayed a significant accumulation of p53 accompanied by p21<sup>Cip1</sup> induction. In contrast, most clones (4/6) carrying the normal Cdk4 alleles lose a functional p53 pathway as determined by either absence of p53 protein, overexpression of p53 due to point mutations or lack of p21<sup>Cip1</sup> induction (Figure 1E). These results indicate that immortal Cdk4<sup>R24C/R24C</sup> cells are resistant to p19<sup>ARF</sup>/p53-induced senescence.

Cdk4<sup>R24C/R24C</sup> MEFs, although immortal, do not show a morphologically transformed phenotype and are not capable of growing under the skin of nude mice. However, these mutant MEFs are susceptible to transformation by Ras oncogenes (Figure 2A). The susceptibility of Cdk4<sup>R24C/R24C</sup> MEFs to Ras transformation is similar to that observed in p15<sup>INK4b-/-</sup> MEFs (Latres *et al.*, 2000) and lower than in INK4a-ARF<sup>-/-</sup> MEFs (Figure 2A). In addition, Cdk4<sup>R24C/R24C</sup> clones grow slower than INK4a-ARF<sup>-/-</sup> cells even after being transformed by Ras oncogenes (Figure 2B). These observations suggest that defects in the p19<sup>ARF</sup>/p53 pathway contribute to Ras susceptibility as well as to Ras transforming activity.

**Table II.** Number of different primary tumors per mouse at death

Tumors per mouse	Males ( <i>n</i> = 61)		Females ( <i>n</i> = 44)		Total ( <i>n</i> = 105)	
0	3	(5%)	4	(9%)	7	(7%)
1	22	(36%)	14	(30%)	35	(33%)
2	21	(34%)	17	(39%)	38	(36%)
3	7	(11%)	7	(16%)	14	(13%)
4	7	(11%)	3	(7%)	10	(10%)
5	1	(2%)	0	(0%)	1	(1%)
Average	1.93		1.81		1.89	

Transformed Cdk4<sup>R24C/R24C</sup> MEFs induced tumors when injected into BALB/c nude mice, although with decreased incidence and longer latency than Ras-transformed INK4a-ARF<sup>-/-</sup> cells (Figure 2C). Whereas all four INK4a-ARF<sup>-/-</sup> clones produced tumors in 15–20 days after injection, only six out of 12 (50%) Cdk4<sup>R24C/R24C</sup> clones gave tumors and with longer latency (25–80 days). No tumors were observed after injection of non-transfected Cdk4<sup>R24C/R24C</sup> cells. To analyze whether neoplastic growth of Cdk4<sup>R24C/R24C</sup> MEFs requires subsequent mutations in the p53 pathway, we tested the cellular levels of p53, p19<sup>ARF</sup> and MDM2 in six subcutaneous tumors derived from Cdk4<sup>R24C/R24C</sup> or INK4a-ARF<sup>-/-</sup> cells. We did not detect overexpression of MDM2 by immunohistochemical analysis. p19<sup>ARF</sup> expression in Cdk4<sup>R24C/R24C</sup> cells was difficult to evaluate due to the low levels of ARF protein in tumors and in surrounding normal cells. p53 expression was very low (~0.1% positive cells) in all tumors regardless of whether they were



**Fig. 3.** (Top panel) Survival curve of  $Cdk4^{R24C/R24C}$  male (gray circles) or female (black circles) mice compared with wild-type littermates (open circles). (A) Representative section of a hemangiosarcoma in the spleen (200 $\times$ ). (B) Metastasis of a hemangiosarcoma in the intestine epithelium (100 $\times$ ).

derived from  $Cdk4^{R24C/R24C}$  or  $INK4a-ARF^{-/-}$  cells. Therefore, it is likely that the  $Cdk4$  R24C mutation can contribute to the tumorigenic properties of Ras-transformed MEFs without additional contribution of the p53 pathway.

#### **Tumor susceptibility in $Cdk4^{R24C/R24C}$ mice**

$Cdk4^{R24C/R24C}$  mice are born at the expected Mendelian ratio, are fertile and develop normally (Rane *et al.*, 1999). However, these mice develop detectable tumors after 8 months and most of them are dead by 16 months of age (Figure 3). Necropsy analysis of a cohort of 105  $Cdk4^{R24C/R24C}$  dead mice showed a wide spectrum of tumors, including malignancies of mesenchymatous origin (67% incidence), epithelial endocrine (55%), epithelial non-endocrine (24%) and, to a lesser extent, hematopoietic malignancies (3%) (Table I). Many mice exhibited multiple tumors of independent origin with an average of almost two tumors per animal (Table II). An independent cohort of 50 mice sacrificed at 14–16 months of age, without external signs of disease, revealed a high incidence of tumors with a similar distribution pattern. However, these mice displayed a reduced incidence of sarcomas and pituitary tumors, suggesting that these malignancies are the major cause of death in  $Cdk4^{R24C/R24C}$  mice (Table I). Heterozygous  $Cdk4^{+/R24C}$  mice also developed the same type of malignancies, although with increased latency

(data not shown) indicating the dominant nature of this mutation.

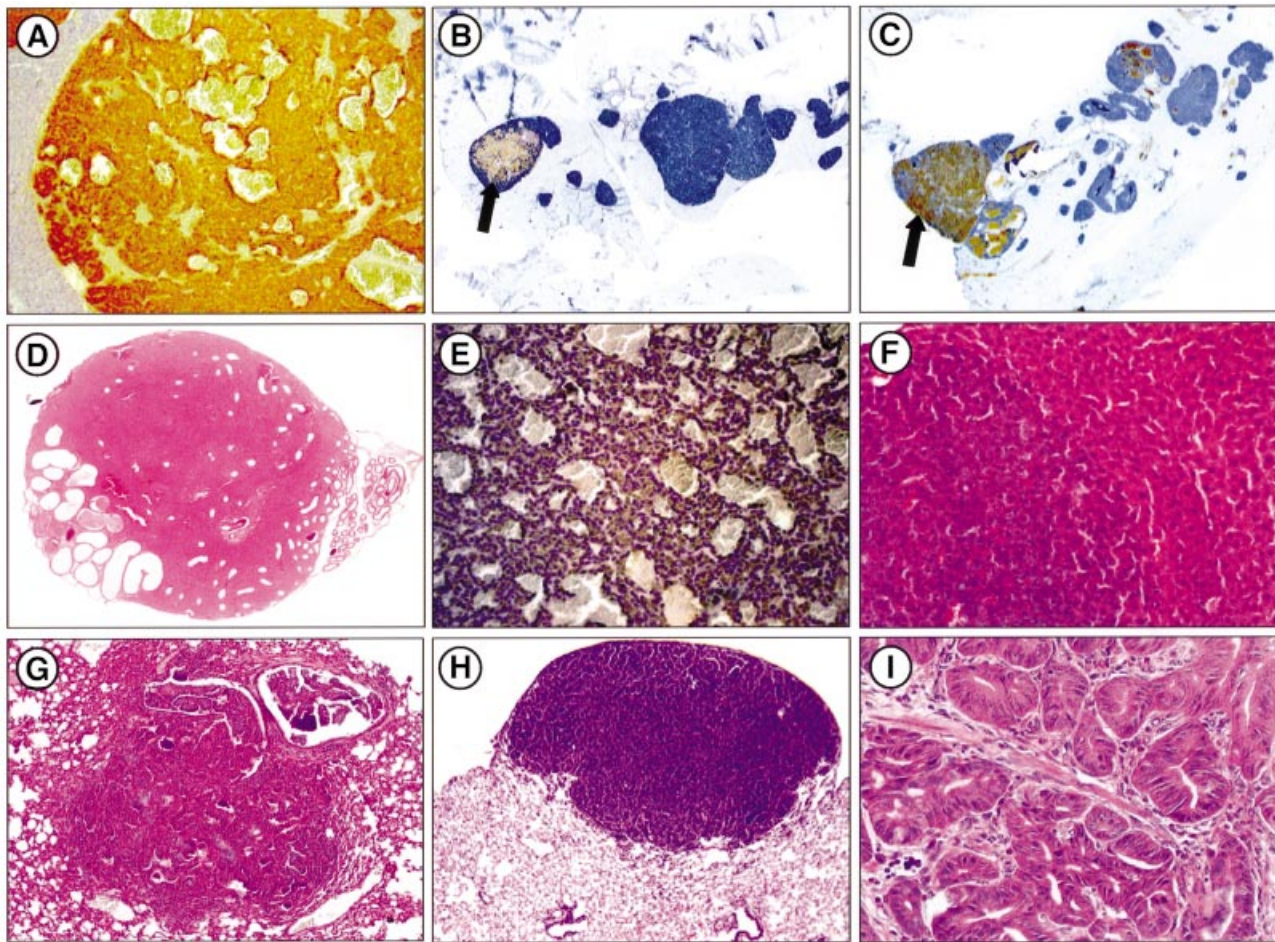
Soft tissue sarcomas developed in 62% of  $Cdk4^{R24C/R24C}$  mice with hemangiosarcoma the most frequent subtype (56% incidence; Figure 3A), a result reminiscent of that observed with  $p15^{INK4b-/-}$  mice (Latres *et al.*, 2000). These tumors appeared at multiple sites (spleen, liver, subcutaneous or deep soft tissues) and were often accompanied by metastasis (54% of the cases; Figure 3B). Among endocrine tumors (Figure 4), those of Leydig cell origin were the most frequent (62% incidence), followed by those derived from pancreatic  $\beta$ -islet cells (31%) and pituitary cells (22%). Leydig cell tumors were preceded by hyperplasia in most of the mice analyzed. These tumors were often bilateral and multifocal but did not metastasize (except in one case) and did not affect the overall survival since male  $Cdk4^{R24C/R24C}$  mice have similar survival rates as female mice (Figure 3).

Pancreatic endocrine tumors were also preceded by severe endocrine hyperplasia. In a panel of 17 pancreas analyzed in detail, we identified 273 small adenomas (16 adenomas/pancreas) ranging from 0.5 to 1.5 mm in diameter. A high proportion of these adenomas (89%) consisted of mostly (>95%) insulin-producing  $\beta$ -cells. However, a few adenomatous islets were made almost exclusively of pancreatic polypeptide (PP)- and glucagon-producing cells (four and one islets, respectively). Moreover, a significant percentage of these pancreatic

**Table III.** Tumors in Cdk4R24C/p53 double mutant mice

	Cdk4 <sup>+/+</sup> ;p53 <sup>-/-</sup> (n = 11)		Cdk4 <sup>+/R24C</sup> ;p53 <sup>-/-</sup> (n = 15)		Cdk4 <sup>R24C/R24C</sup> ;p53 <sup>-/-</sup> (n = 41)	
Average age at death	4.7 months <sup>a</sup>		3.9 months		3.3 months <sup>a</sup>	
Tumor type						
lymphoma	9	(82%)	11	(73%)	21	(51%)
sarcoma	2	(18%)	6	(40%)	20	(49%)
teratoma	0	(0%)	0	(0%)	4	(10%)

<sup>a</sup>Statistically significant ( $P < 0.001$ ) using the Student's *t*-test.



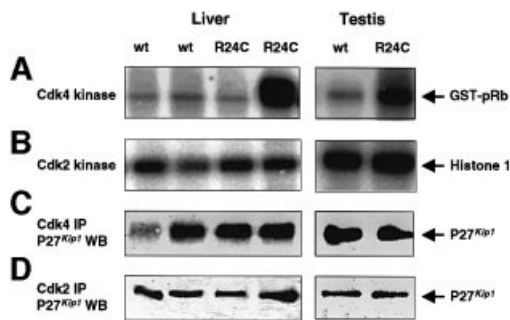
**Fig. 4.** Endocrine and epithelial non-endocrine tumors in Cdk4<sup>R24C/R24C</sup> mice. (A) Pancreatic endocrine tumor positive for insulin immunostaining (100 $\times$ ). (B–C) Adenomatous pancreatic islets showing cells positive for (B) insulin (blue) and glucagon (brown cells indicated by an arrow) or (C) insulin and PP (brown cells indicated by an arrow) (100 $\times$ ). (D) Leydig cell tumor with extension to the epididymis (200 $\times$ ). (E) Adenocarcinoma of the adenohypophysis immunoreactive for prolactin (200 $\times$ ). (F) Liver adenoma (200 $\times$ ). (G) Lung adenocarcinoma (100 $\times$ ). (H) Alveolar bronchiolar adenoma (400 $\times$ ). (I) Intestinal adenoma with *in situ* carcinoma (400 $\times$ ).

adenomas (~10%) contained a mixture of endocrine cells including  $\beta$ ,  $\alpha$  and PP cells (Figure 4A–C). These results indicate that improper Cdk4 regulation can affect the proliferative properties of a variety of endocrine pancreatic cells. However, no hyperplastic somatostatin-producing delta cells have been identified in the pancreas of Cdk4<sup>R24C/R24C</sup> mice.

Most of the pituitary tumors observed in Cdk4<sup>R24C/R24C</sup> mice were adenomas with a variety of hormone expression patterns. However, ~15% of these tumors appeared to be carcinomas, most of them positive for prolactin

expression. Bronchio-alveolar adenomas and adenocarcinomas of the lung were the third most common tumor in Cdk4<sup>R24C/R24C</sup> mice, a result reminiscent of those found in p18<sup>INK4c</sup><sup>-/-</sup>; p27<sup>Kip1</sup><sup>-/-</sup> double mutant mice (Franklin *et al.*, 2000). Finally, adenoma or hyperplasia of the mammary gland, hepatocytes, salivary gland, adrenal subcapsular cells, prostatic and endometrial cells (1–7% incidence) were also observed.

Other common pathologies detected in Cdk4<sup>R24C/R24C</sup> mice included extramedullary hematopoiesis in spleen and liver (43% incidence) associated with myeloid metaplasia



**Fig. 5.** (A) Cdk4 and (B) Cdk2 kinase activity in representative liver and testis samples from Cdk4<sup>R24C/R24C</sup> mice. Kinase activity was assayed after immunoprecipitation with Cdk4 or Cdk2 antibodies. R24C lanes depict examples of one out of four tissues in which we did not observe increased Cdk4 kinase activity and of two out of eight tissues in which the Cdk4 kinase activity was elevated. In none of the samples, we observed significantly increased Cdk2 activity. (C and D) Distribution of p27<sup>Kip1</sup> in complexes formed with (C) Cdk4 or (D) Cdk2 in the above samples. IP, immunoprecipitation; WB, western blot. The migration of the Cdk4 and Cdk2 substrates, GST-pRb and Histone H1, respectively and of p27<sup>Kip1</sup> is indicated by arrows.

(13%), lymphoid hyperplasia in the spleen (6%) and in the lymph nodes (13%), glomerular cysts (27%), chronic nephropathy (9%) and ductal ectasia of mammary glands (1%). Many of these pathologies have also been reported in mice deficient for p15<sup>INK4b</sup> and/or p18<sup>INK4c</sup> inhibitors (Latres *et al.*, 2000). In addition, organomegaly was common in male genital annexed glands such as seminal vesicles, coagulating glands and prostate, without evidence of histological abnormalities.

#### **Absence of INK4a, INK4b, ARF and p53 alterations in Cdk4R24C tumors**

Alterations of the genes encoding p16<sup>INK4a</sup>, p15<sup>INK4b</sup> and p19<sup>ARF</sup> proteins are frequently found in rodent tumors (Ruas and Peters, 1998). Whereas these loci are commonly deleted or inactivated by promoter hypermethylation, point mutations are rare. To investigate whether these alterations also occur in Cdk4<sup>R24C/R24C</sup> tumors, we analyzed deletion of the individual p16<sup>INK4a</sup>, p15<sup>INK4b</sup> and p19<sup>ARF</sup> genes as well as the methylation status of their respective promoters. Southern analysis of DNA tumor samples did not show deletions in any of these three genes in a panel of 31 tumors (13 sarcomas, seven Leydig cell tumors, seven pituitary tumors and four hepatocellular tumors). Similarly, no methylation of their respective promoters was detected in any of these samples (data not shown).

The status of the p53 gene was also studied in Cdk4<sup>R24C/R24C</sup> tumors. Exons 5–9 of the murine p53 gene from 17 tumor samples, including representative epithelial tumors and sarcomas, were amplified by PCR using intronic primers. No point mutations were found in any of the samples after amplification and DNA sequencing (data not shown). To further analyze the integrity of the p53 pathway, we studied the protein levels of MDM2, p19<sup>ARF</sup> and p53 in these tumors. Western blot and immunohistochemical detection of these proteins was performed in a representative number of sarcomas (18 samples), Leydig cell tumors (11 samples), pancreatic endocrine adenomas (10 samples), and tumors of the liver (four samples),

lung (four samples) and pituitary (five samples). Overexpression of MDM2 was only detected in two out of 11 (18%) pancreatic endocrine adenomas. The presence of p19<sup>ARF</sup> was difficult to evaluate due to the low level of expression in most organs. However, when the comparison was possible, p19<sup>ARF</sup> levels in tumor cells were equivalent to those of the corresponding normal cells. Similarly, no differences were detected in the levels of p53 in any of the tumors analyzed. These results suggest that loss of Cdk4 regulation by INK4 inhibitors is sufficient to induce a variety of tumors *in vivo*, not requiring cooperation by a mutated p19<sup>ARF</sup>/p53 pathway, at least in most cases.

#### **Cdk4 and Cdk2 activity and p27<sup>Kip1</sup> complexes in Cdk4R24C tissues**

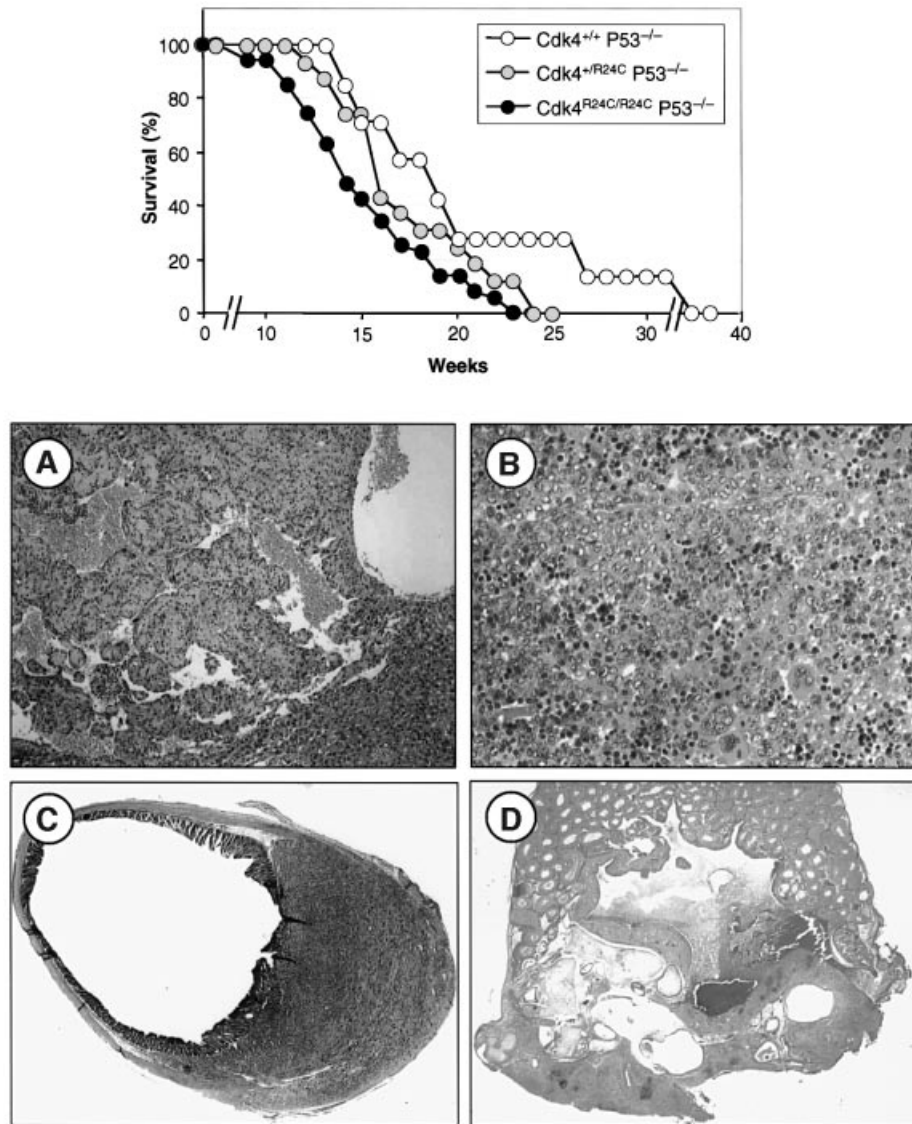
The high susceptibility to tumor development in homozygous as well as heterozygous Cdk4<sup>R24C</sup> mice suggests that the R24C mutation has a dominant effect in the fate of several cellular lineages. This mutation makes the Cdk4 kinase resistant to INK4 inhibition (Wolfel *et al.*, 1995) and results in pRb hyperphosphorylation in cultured MEFs (Rane *et al.*, 1999). We tested Cdk4 and Cdk2 kinase activity in tissues derived from wild-type and Cdk4<sup>R24C/R24C</sup> mice. Higher Cdk4 activity was observed in most, but not all (eight out of 12), tissues derived from Cdk4<sup>R24C/R24C</sup> mice (Figure 5). In contrast, no significant increases in Cdk2 activity were observed (Figure 5). Next, we assessed whether the Cdk4<sup>R24C</sup> mutation affected the redistribution of p27<sup>Kip1</sup> between Cdk4 and Cdk2 complexes. As illustrated in Figure 5, we did not observe significant differences in the distribution of p27<sup>Kip1</sup> in Cdk4<sup>R24C/R24C</sup> cells when compared with those derived from wild-type mice.

#### **Cooperation between Cdk4R24C and p53 deficiency**

The low frequency of ARF/MDM2/p53 mutations in Cdk4<sup>R24C/R24C</sup> tumors does not preclude the possibility of cooperation between Cdk4 deregulation and p53 mutations. To examine this question, we crossed Cdk4<sup>R24C/R24C</sup> mutant mice with p53<sup>-/-</sup> knock-out animals. Double mutant Cdk4<sup>R24C/R24C</sup>;p53<sup>-/-</sup> or Cdk4<sup>R24C/R24C</sup>;p53<sup>-/-</sup> mice die before reaching 4 months of age, significantly earlier than their Cdk4<sup>R24C/R24C</sup>;p53<sup>-/-</sup> littermates (Figure 6; Table III). These mice display an increased number of sarcomas (mainly hemangiosarcomas and leiomyosarcomas) with shorter latency. The increased incidence of these tumors probably accounts for the lower incidence of diffuse large cell lymphomas in the Cdk4<sup>R24C/R24C</sup>;p53<sup>-/-</sup> and Cdk4<sup>R24C/R24C</sup>;p53<sup>-/-</sup> mice (Table III). Moreover, a significant fraction of these double mutant mice (10%) develop immature teratomas, a tumor not found in any of their single mutant siblings, although a low incidence of teratomas (<2%) has been described previously in p53<sup>-/-</sup> mice (Jacks *et al.*, 1994). These results suggest that these key regulators exert growth control at different threshold levels in different cell types.

#### **Cooperation between the Cdk4R24C mutation and p27<sup>Kip1</sup> deficiency**

Cdk4<sup>R24C/R24C</sup> mice develop pituitary tumors with high penetrance, a phenotype also observed in mice targeted for other components of the INK4/Cdk4/Rb pathway, pRb<sup>+/-</sup>

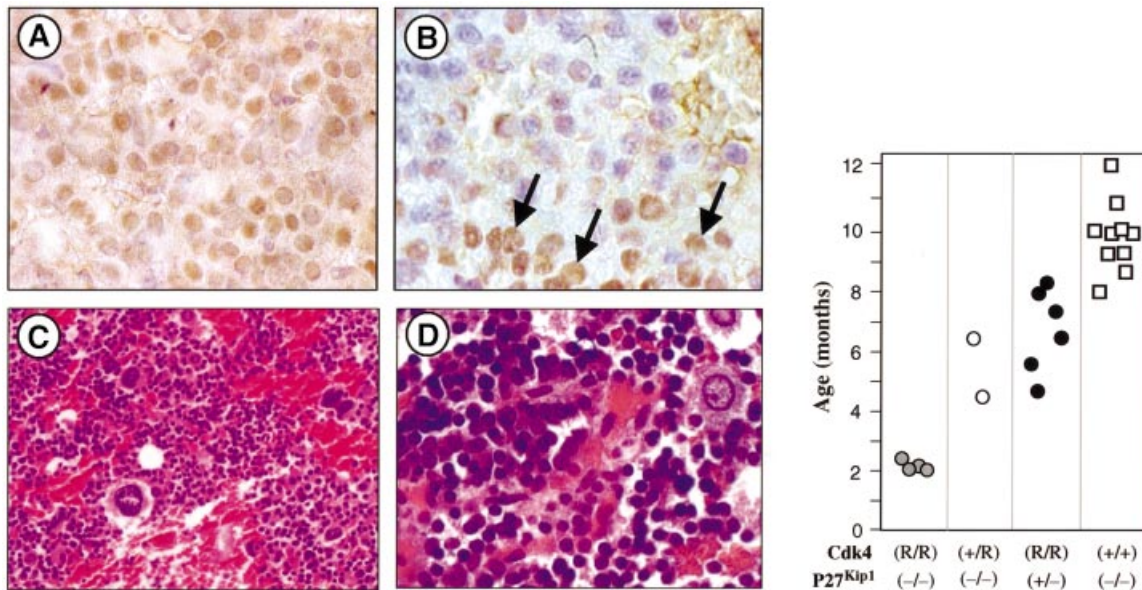


**Fig. 6.** Cooperation between the Cdk4R24C allele and p53 deficiency. (Top panel) Tumor-free survival curve of p53-deficient mice carrying one or two Cdk4R24C alleles. (A–D) Representative sections of tumors obtained from Cdk4<sup>R24C/R24C</sup>;p53<sup>-/-</sup> double mutant mice. (A) Hemangiosarcoma located in the liver (100 $\times$ ). (B) Typical diffuse large cell lymphoma in spleen (400 $\times$ ). (C) Leiomyosarcoma with intermediate differentiation spreading to the large intestine/rectum area (200 $\times$ ). (D) Teratoma in the testis formed by mature structures such as cysts lined by columnar or epidermoid epithelium (center), bone (right) and cartilage (left), and a large immature area (200 $\times$ ).

and p18<sup>INK4c</sup><sup>-/-</sup>. Moreover, mutations in Cip/Kip inhibitors such as p27<sup>Kip1</sup> also result in pituitary tumors (reviewed in Malumbres *et al.*, 2000a). In fact, concomitant loss of p18<sup>INK4c</sup><sup>-/-</sup> and p27<sup>Kip1</sup> results in significant reduction of tumor latency, thus suggesting cooperation between INK4 and Cip/Kip inhibitors (Franklin *et al.*, 1998). To determine whether p27<sup>Kip1</sup> plays a role in the development of pituitary tumors in Cdk4<sup>R24C/R24C</sup> mice, we examined p27<sup>Kip1</sup> expression by immunohistochemical staining. Wild-type pituitary tissue shows p27<sup>Kip1</sup> immunoreactivity in most endocrine cells from the adenohypophysis and the *pars intermedia*. However, p27<sup>Kip1</sup> staining is either lost or severely reduced in most (9/10) Cdk4<sup>R24C/R24C</sup> pituitary tumors analyzed (Figure 7).

Next, to assess the extent of the cooperation between the Cdk4R24C mutation and loss of p27<sup>Kip1</sup> in tumor development, we crossed Cdk4<sup>R24C/R24C</sup> mice with animals

deficient in this Cip/Kip inhibitor. Pituitary tumors in Cdk4<sup>R24C/R24C</sup> mice were detectable at 14–16 months of age. Likewise, p27<sup>Kip1</sup>-deficient mice died of pituitary tumors with an average latency of 10 months (Figure 7). In contrast, Cdk4<sup>R24C/R24C</sup>;p27<sup>Kip1</sup><sup>-/-</sup> double mutant mice died of pituitary tumors with an average latency of ~2 months (Figure 7). These tumors, unlike those found in Cdk4<sup>R24C/R24C</sup> mice, were mainly undifferentiated neoplasias of the *pars intermedia*, similar to pituitary tumors arising in pRb<sup>+/-</sup>; p27<sup>Kip1</sup><sup>-/-</sup> mutants (Park *et al.*, 1999). Cdk4<sup>+/R24C</sup>;p27<sup>Kip1</sup><sup>-/-</sup> and Cdk4<sup>R24C/R24C</sup>;p27<sup>Kip1</sup><sup>+/-</sup> mice also developed pituitary tumors with complete penetrance. However, these strains displayed increased latency (5.6 months in Cdk4<sup>+/R24C</sup>;p27<sup>Kip1</sup><sup>-/-</sup>; 6.7 months in Cdk4<sup>R24C/R24C</sup>;p27<sup>Kip1</sup><sup>+/-</sup>). These results, in agreement with the biochemical data shown in Figure 5, suggest that tumor development induced by the Cdk4R24C



**Fig. 7.** Cooperation between the Cdk4<sup>R24C</sup> mutation and loss of p27<sup>Kip1</sup> in the development of pituitary tumors. (A) p27<sup>Kip1</sup> expression in wild-type adenohypophysis. Most nuclei from endocrine cells are positive (1000 $\times$ ). (B) Cdk4<sup>R24C/R24C</sup> adenoma of the adenohypophysis showing areas of positive cells (arrows) and tumoral regions negative for p27<sup>Kip1</sup> immunoreactivity (1000 $\times$ ). (C and D) Undifferentiated tumor of the *pars intermedia* in a Cdk4<sup>R24C/R24C</sup>;p27<sup>Kip1</sup><sup>-/-</sup> mouse [hematoxylin and eosin staining; (C) 400 $\times$ ; (D) 1000 $\times$ ]. (Right panel) Latency of pituitary tumors in animals derived from the crosses between Cdk4<sup>R24C/R24C</sup> and p27<sup>Kip1</sup><sup>-/-</sup> mice. The y-axis represents the age of the mice at death in the different genotypes.

mutation is not due to increased sequestration of p27<sup>Kip1</sup>, at least in pituitary cells.

## Discussion

Available evidence has suggested that the p19<sup>ARF</sup>/p53 rather than the INK4/pRb pathway is primarily responsible for inducing senescence (Harvey and Levine, 1991; Kamijo *et al.*, 1997). However, concomitant deletion of the three members of the Rb family of proteins also confers immortality to these cells since MEFs deficient for pRb, p107 and p130 do not sustain functional mutations in the p53 pathway even upon prolonged passage in culture (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). Here, we show that expression of a mutant Cdk4 whose kinase activity cannot be down-regulated by INK4 inhibitors is also sufficient to confer immortality to MEFs. MEFs derived from Cdk4<sup>R24C/R24C</sup> embryos grow significantly faster than wild-type MEFs and have properties similar to those observed in MEFs lacking the three Rb family members. Moreover, none of these properties require mutations in the p19<sup>ARF</sup>/p53 pathway. Thus, Cdk4 activity, when resistant to INK4 inhibition, is sufficient to inactivate the entire Rb protein family, at least during immortalization of MEFs in culture. These results suggest that the INK4 proteins are the primary effectors responsible for maintaining the activity of pRb family members during senescence. Whether this activity requires all or a subset of INK4 inhibitors, remains to be determined. However, since concomitant deletion of p15<sup>INK4b</sup> and p18<sup>INK4c</sup> proteins does not result in MEF immortalization (Latres *et al.*, 2000), we conclude that proper control of Cdk4 activity during senescence must require the coordinated action of at least three members of the INK4 protein family.

Cdk4<sup>R24C/R24C</sup> MEFs are also susceptible to transformation by Ras oncogenes, a property shared by MEFs defective in all Rb family members (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). Interestingly, this property can be attributed to the specific action of single INK4 proteins since we have previously shown that p15<sup>INK4b</sup>-deficient MEFs can be transformed by Ras oncogenes (Latres *et al.*, 2000; Malumbres *et al.*, 2000b). In contrast, loss of either p18<sup>INK4c</sup> or p19<sup>INK4d</sup> proteins does not confer this property (Latres *et al.*, 2000; Zindy *et al.*, 2000). These results indicate that certain phenotypic consequences of the unregulated Cdk4 activity, such as susceptibility to Ras-induced transformation, can be independently controlled by specific INK4 inhibitors.

Deregulation of G<sub>1</sub>/S cell cycle kinases is one of the most frequent events in human cancer. Cdk4<sup>R24C/R24C</sup> mice develop a wide spectrum of tumors. Indeed, the intense tumor susceptibility of these mutant mice contrasts with the collective data showing that disabling single INK4 family members does not dramatically increase the rate of spontaneous tumor development (Roussel, 1999; Malumbres *et al.*, 2000a). These observations suggest that INK4 proteins have significant overlapping properties in most cell types. This might explain not only the increased incidence of tumor formation in Cdk4<sup>R24C/R24C</sup> mice, but also the significantly wider spectrum of tumor types. Interestingly, many of the tumors observed in Cdk4<sup>R24C/R24C</sup> mice originate from endocrine cells of the pancreas, pituitary or testis, the very same cell types that have been previously shown to have limited proliferative properties in mice lacking Cdk4 expression (Rane *et al.*, 1999; J.Martin, S.L.Hunt, P.Dubus, R.Sotillo, M.Malumbres, S.Ortega and M.Barbacid, in preparation). These results suggest that Cdk4 plays a central role in controlling the proliferation of these endocrine cells.



Some of the tumors found in Cdk4<sup>R24C/R24C</sup> mice, such as soft tissue sarcomas, reproduce findings with human tumors where oncogenic amplification and overexpression of Cdk4 has commonly been reported (Khatib *et al.*, 1993; Wei *et al.*, 1999; Wunder *et al.*, 1999). In these tumors, Cdk4 is frequently co-amplified with other genes including MDM2 (Khatib *et al.*, 1993; Wunder *et al.*, 1999). Cdk4 overexpression has also been reported in other human tumors such as breast cancer, gliomas or meningiomas (He *et al.*, 1994; Schmidt *et al.*, 1994; Weber *et al.*, 1997; An *et al.*, 1999). Surprisingly, Cdk4R24C mutant mice do not develop melanomas, the tumor type observed in humans with the same genotype. Recent studies in which these mutant mice have been exposed to various skin carcinogenesis protocols have resulted in the frequent appearance of invasive melanomas (Sotillo *et al.*, 2001). These results indicate that human melanoma may result from a combination of cell cycle mutations followed by additional genotoxic events induced by environmental carcinogens.

The most frequent tumor types observed in Cdk4<sup>R24C/R24C</sup> mice match those previously found in mice deficient in INK4 inhibitors, albeit with lower incidence (Franklin *et al.*, 1998; Latres *et al.*, 2000; Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001). For instance, hemangiosarcoma is the predominant tumor type in p15<sup>INK4b</sup>-deficient mice and pituitary tumors are predominant in p18<sup>INK4c</sup> null mice. Hematopoietic malignancies and other minor pathologies such as renal cysts, mammary gland ectasia or pancreatic  $\beta$ -cell hyperplasia were also described in the p15<sup>INK4b</sup>-p18<sup>INK4c</sup> double knockout or in the single mutants (Latres *et al.*, 2000). Of particular interest are pituitary tumors which in addition to p18<sup>INK4c</sup> null mice have been found in other cell cycle mutant mice such as pRb<sup>+/-</sup> and p27<sup>Kip1</sup><sup>-/-</sup> (Jacks *et al.*, 1992; Hu *et al.*, 1994; Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996; Franklin *et al.*, 1998; Latres *et al.*, 2000). These tumors, however, arise mostly from the *pars intermedia* [75% in the case of the pRb mutant mice (Nikitin *et al.*, 1999) and 98% in the case of p18<sup>INK4c</sup><sup>-/-</sup> mice (Latres *et al.*, 2000)], whereas those expressing a mutated Cdk4R24C protein are mostly of adenohypophysis origin (80%). However, when pRb is conditionally deleted in specific cells (IRBP-Cre;Rb/floxed mice), all the pituitary tumors develop from the adenohypophysis (Vooijs and Berns, 1999). Since Cdk4 acts downstream of p18<sup>INK4c</sup> and upstream of pRb, these results illustrate the delicate balance that these proteins interplay in regulating cell proliferation in cells of common ectodermic origin.

Cdk4R24C tumors are quite similar to those found in mice deficient for p53 (hemangiosarcomas and lung adenocarcinomas) and in mice heterozygous for pRb and p53 (pituitary adenomas, pancreatic islet cell adenocarcinomas, hemangiosarcoma and lymphomas) (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Williams *et al.*, 1994; Harvey *et al.*, 1995). We have not found mutations in the p53 tumor suppressor gene in a wide panel of representative Cdk4<sup>R24C/R24C</sup> tumors, indicating that they arise without cooperating p53 mutations. However, when we expressed the Cdk4R24C mutant protein in a p53<sup>-/-</sup> background, the incidence of sarcomas and teratomas increased significantly, illustrating the different

requirements of distinct cell types to undergo neoplastic transformation.

Previous studies have illustrated the cooperation between the INK4/pRb and the p27<sup>Kip1</sup> pathways in inducing pituitary tumors (Franklin *et al.*, 1998, 2000; Park *et al.*, 1999). Interestingly, loss of p27<sup>Kip1</sup> reverts some of the defects caused by loss of Cdk4 (Tsutsumi *et al.*, 1999) and cyclin D1 (Geng *et al.*, 2001; Tong and Pollard, 2001). These observations are consistent with the hypothesis that Cdk4-cyclin D complexes may play a role in sequestering p27<sup>Kip1</sup> during G<sub>1</sub>/S transition to activate the Cdk2 kinase (Sherr and Roberts, 1999). According to this hypothesis, lack of INK4-mediated inhibition of Cdk4 in Cdk4<sup>R24C/R24C</sup> mice should increase the availability of Cdk4 molecules to form Cdk4-Cyclin D-p27<sup>Kip1</sup>-ternary complexes. However, our results are not consistent with this hypothesis. First of all, we have not observed significant p27<sup>Kip1</sup> redistribution into Cdk4-Cyclin D complexes in tissues expressing the Cdk4 R24C kinase. Moreover, pituitary tumors of Cdk4<sup>R24C/R24C</sup> mice invariably lose p27<sup>Kip1</sup> expression. Finally, and perhaps most compelling, is the dramatic decrease in tumor latency observed in Cdk4<sup>R24C/R24C</sup>;p27<sup>Kip1</sup> double mutant mice. Therefore, although our results do not preclude a role of Cdk4 in sequestering p27<sup>Kip1</sup>, they suggest that loss of INK4 inhibition by the Cdk4 R24C mutation is more likely to increase Cdk4-dependent pRb phosphorylation than sequestration of p27<sup>Kip1</sup>.

Proliferative disorders such as cancer are recognized as diseases of the cell cycle. G<sub>1</sub>/S-phase control mechanisms are aberrant in most human tumors and INK4/Cdk4/Rb regulation seems to be a central step in this control (Sherr, 2000). These results have validated the utilization of the cell cycle as a target for drug discovery (Brooks and La Tangué, 1999), underscoring the urgent need to develop specific inhibitors that downregulate this pathway in tumor cells. An ideal target for pharmacological intervention would be essential for growth or survival of tumors but dispensable in normal tissues. Cdk4 fulfils these requirements since Cdk4 is not essential for mouse development (Rane *et al.*, 1999; Tsutsumi *et al.*, 1999). Yet, loss of just one of its multiple regulatory mechanisms due to lack of INK4 binding is sufficient to elicit a wide variety of tumors in many cell types. If disabling INK4 inhibitors is a hallmark of many cancers (Ruas and Peters, 1998; Sherr, 2000), then the most efficacious treatment would be to restore their effect by therapeutic inhibition of Cdk4 activity, an approach more readily applicable than restoring INK4 function. Cdk4<sup>R24C/R24C</sup> mice should provide an invaluable tool to test the anti-tumor properties of inhibitors of Cdk4 and possibly Cdk2 kinase activity, as well as future inhibitors of Cdk4-Cyclin D complexes, and thus help to speed up the process of translational research.

## Materials and methods

### Mice

Cdk4<sup>R24C/R24C</sup> mice expressing an endogenous Cdk4R24C protein were obtained by gene-targeting techniques as described previously (Rane *et al.*, 1999). Cdk4-deficient mice (Cdk4<sup>neo/neo</sup>) were also reported (Rane *et al.*, 1999). Cdk4<sup>R24C/R24C</sup> and Cdk4<sup>neo/neo</sup> mice were maintained in a mixed 129/Sv  $\times$  CD-1 background. Mice deficient for the p53 or p27<sup>Kip1</sup> tumor suppressor genes have been published previously (Jacks *et al.*, 1994; Fero *et al.*, 1996). Athymic BALB/c nu/nu mice were purchased

from Charles River Laboratories. All mice were maintained according to institutional guidelines.

### MEF assays

MEFs were isolated by standard techniques and maintained in a 3T3 scheme with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Immortalization and proliferation assays were performed as described (Latres *et al.*, 2000). The status of the p53 pathway was assessed essentially as described by Pantoja and Serrano (1999). Focus assays were performed by calcium phosphate-mediated transfection of MEFs with pAL8, an expression plasmid carrying an oncogenic H-*ras* allele under the control of its own promoter (Latres *et al.*, 2000). Transformed clones (foci) were isolated, expanded and injected into nude mice ( $5 \times 10^5$  cells per injection). Tumor volume ( $V$ ) was calculated using the formula  $V = (d^2 \times D)/2$  where  $d$  is the minor diameter and  $D$  the major diameter of the tumor.

### Necropsy and pathological analysis

Mice were sacrificed at any sign of disease and normal or pathological tissue samples were recovered for histological and molecular analysis. A group of 50 apparently healthy Cdk4<sup>R24C/R24C</sup> mice was sacrificed at the age of 16 months. Formalin-fixed pituitary samples were stained with antibodies against Adrenocorticotropic hormone, follicle-stimulating hormone, growth hormone, prolactin, luteinizing hormone and thyroid-stimulating hormone (kindly provided by A.F.Parlow, NHPP, NIDDK). Endocrine tumors of the pancreas were stained with antibodies against insulin, glucagon, somatostatin (Dako Corp.) and PP (Monosan). Deletion or hypermethylation of the *p16<sup>INK4a</sup>*, *p15<sup>INK4b</sup>* and *p19<sup>ARF</sup>* genes was studied as described (Malumbres *et al.*, 1997; Meléndez *et al.*, 2000). To analyze the presence of mutations in p53, we previously obtained the sequence of the mouse introns 4–8. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession No. AF367373. We then designed specific primers to amplify exons 5–9 of p53 and the presence of mutations in the tumor samples was scored by direct sequencing of amplified products. Sequence (5'–3') of primers is as follows: mp53-5F, 5'-TACTCTCCT-CCCCTCAATAAG-3'; mp53-I6R, 5'-CGGGTTGCTAGAAAGTCA-ACA-3'; mp53-I6F, 5'-TGC-CGAACAGGT GGAATATC-3'; mp53-I7R, 5'-TGGAACAGAAA-CAGGCAGAA-3'; mp53-I8F, 5'-TCTCGGGGT-CTGTAACTG-3'; mp53-9R, 5'-GGATCTTGAGGGTCAAATACTC-TCC-3'. Expression of p27<sup>Kip1</sup> (clone DCS-72.F6, Neomarkers), p53 (NCL-p53-CM5p, Novocastra), p19<sup>ARF</sup> (SC-7403 from Santa Cruz Biotechnology, or R562 from Abcam) and MDM2 (SC-965, Santa Cruz Biotechnology) was analyzed by immunohistochemistry in paraffin sections of the tumors using the indicated antibodies.

### Immunoprecipitation, kinase and western blot analysis

Tissue samples or cultured cells were lysed in a solution containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% nonidet P-40 and protease inhibitors. For immunoprecipitation, cell lysates were incubated with 5 µg of anti-Cdk4 or anti-Cdk2 antibody (Santa Cruz Biotechnology) and 200 µl of protein A beads on a rotating wheel at 4°C for 2 h. Cdk4 kinase assays were performed with 100 ng of glutathione S-transferase (GST)-Rb (Santa Cruz Biotechnology) as a substrate. Histone H1 (1 µg per reaction; Roche Biochemicals) was used as a Cdk2 substrate. Kinase assays were performed in a solution containing 20 mM Tris-HCl pH 8, 1 mM EGTA, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 25 µM cold ATP and 10 µCi of [<sup>32</sup>P]ATP. Mixtures were incubated at 30°C for 30 min, and the reactions were stopped by the addition of SDS loading buffer. Reaction mixtures were run on a 12% SDS-polyacrylamide gel that was stained with Coomassie Blue. Quantification of phosphorylated proteins was performed on a PhosphorImager (Molecular Dynamics). Gels stained with Coomassie Blue were also scanned by densitometry to quantify the exact amount of protein present on the gel by NIH image software. For western blots, whole cell extracts or immunoprecipitates, prepared as above, were separated on a 12% SDS-PAGE gel. Following electrophoresis, proteins were transferred to nitrocellulose before incubation with the following antibodies: Cdk4 (C-22), Cdk2 (M2) and p21<sup>Cip1</sup> (C-19, all from Santa Cruz Biotechnology), p53 (CM-5, Novocastra) and p27<sup>Kip1</sup> (DCS-72.F6, Neomarkers). The antibodies were detected using anti-mouse or anti-rabbit Ig horseradish peroxidase linked whole antibody and visualized by the enhanced chemiluminescent detection system (Amersham).

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